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Debugging systematic bacteriology

THE International Committee on Systematic Bacteriology (ICSB) has reason to be pleased with itself. For there is shortly to appear in the *International Journal of Systematic Bacteriology* the ICSB's new Approved List of Bacterial Names, a list that will include only about 2500 of the 30,000 bacterial species that have been named over the years. Only a handful of those species that have been excluded are likely to worm their way back onto the approved list since, by the standards laid down by the ICSB, their alleged identity is unacceptable.

The identification of bacteria dates back, in principle, to 1753 and the *Species Plantarum* of Linnaeus although in practise it was only from the middle of the nineteenth century that identification of species started in earnest. The problem that has grown ever since then is that by modern standards the vast majority of named species were given descriptions that were totally inadequate for them ever to be recognised again. Furthermore, for very few of the 30,000 alleged species was there a type culture — that is, an authentic specimen of the original beast — with which any new specimen could be compared.

In these circumstances it had become almost impossible for anyone to identify, unambiguously, a new species of bacterium. To do so meant laboriously consulting old and inadequate literature. It meant sifting through hundreds of names, scores of which might apply to the same species. Experts would disagree about which names applied to the same species and which single name to opt for. The competing names often lingered on in the literature with each in turn being overturned or revived as new evidence turned up. And when the issue might have been settled by re-examining the species themselves, cultures either did not exist, were of dubious authenticity or had clearly become outgrown by contaminating bacteria.

It was because of this situation that the International Committee on Systematic Bacteriology of the International Association of Microbiological Societies was persuaded to undertake the formidable task of hacking away the dying, decaying and merely dubious foliage in the hopeless jungle of bacterial nomenclature. After years of committee work, the experts have decided that there are only about 2500 species of bacteria that are currently recognised by most bacteriologists and

which possess type strains or equivalent type material. From now on bacteriologists need only consider these approved species and, of course, any that are subsequently added to it. Additions will be carefully controlled by the requirement for better descriptions and designations of type strains than in the past. More controversially, new names will only be acceptable once they have been published in the *International Journal of Systematic Bacteriology*.

Had the last requirement amounted to a monopoly of publication there would have been room to criticise it both on principle and as a workable proposition. The committee, however, have acknowledged that publication of the full details of newly named bacteria can be in any scientific journal or book whilst stating that they will not validate any name until it has been announced (with reference to the full paper) in the *International Journal of Systematic Bacteriology*. This makes good sense since it will mean that any initial search through past names can henceforth be made in a single journal.

It is also good that the Judicial Commission of the ICSB is willing to consider proposals to re-admit bacteria that have been excluded from the approved list. No gathering of experts, however conscientious, could have hoped to make all the correct decisions and doubtless a small number of old names will get back on the list. The Judicial Commission should be firm but sympathetic to anyone who is willing to champion the re-appraisal of their pet species provided that they are prepared to produce adequate evidence, a modern description and a satisfactory type strain.

This attempt of bacteriologists to put their house in order has implications for other biologists. The great conservatism of nomenclaturalists is well known. The idea of abandoning the principles of priority is anathema to them. But when the application of the principle causes more instability than the stability it is intended to preserve, then it is indeed time to make a fresh start. The plan adopted for bacteria may be directly applicable to only a few other groups of living organisms. But the principles followed in the plan may be of wider use when the nomenclature of other groups of living creatures, or of other scientific entities (eg genes, enzymes, ecosystems) comes to need revision. □

New UK Select Committee needs more attack

ONE of the first trials of the new UK select committee system took place last week, when the House of Commons Select Committee on Energy confronted the Secretary of State for Energy, Mr David Howell, and his junior ministers, on the government's recently announced nuclear policy. The Secretary of State undoubtedly won the battle.

Not that that was entirely due to the integrity of Mr Howell's case. While Mr Howell is a thoughtful minister, and well-briefed by his large department, there are inevitably weak points in his thinking and lacunae in his policy; and it is the job of the Select Committee to bring them out.

For example, what of the accusation that if the £10-12 billion of government money allocated to the nuclear plan had instead been allocated to existing and proven conservation technologies, three times the primary energy value of the reactors could have been saved? On a recent television programme, Mr Norman Lamont, the minister responsible for nuclear affairs, answered this with a remark about it being impossible to control the needs and desires of energy consumers, missing the point completely that both the nuclear plan and a conservation plan are matters of direct

government spending, as when the gas-cookers of Britain were converted, at government expense, to North Sea gas.

Further, it is clear that one of the principle objectives of ordering ten new nuclear reactors is to assure the strength of the British nuclear industry. But after the long history of set-back with the advanced gas-cooled reactor, and the loss of many highly-trained staff, is the industry capable of carrying the orders out?

The new arrangement of select committees, which parallels the arrangement of government departments and has hence eliminated the useful form of the Select Committee on Science and Technology, was intended to give MPs a more direct basis of attack on government policy. But on last week's performance it seems unlikely to have that effect. More of those serving on the committees should be prepared to do some hard homework, and the committees should be more prepared to organise themselves to attack and expose the weak points in government thinking. Neither the homework (except in one notable case) nor the organisation were particularly evident at the Select Committee on Energy last week. □



United States

OSHA looks at occupational regulations for recombinant DNA use

REFLECTING the increasingly rapid movement of recombinant DNA techniques from the research laboratory to the industrial production line, occupational health agencies in Washington are beginning to discuss how they should handle any health hazards that may be involved.

Dr Eula Bingham, Assistant Secretary of Labour and head of the Occupational Safety and Health Administration (OSHA), has suggested that an expert panel be set up to address the hazards of DNA use "particularly as they apply to potential occupational exposures".

In a letter to Dr Donald Fredrickson, Director of the National Institutes of Health, Dr Bingham says she supports the system of voluntary compliance by private industry with the NIH guidelines which, after a period of public comment, was implemented last week.

But she adds that the agency feels that "more formalised enforcement of procedures and rules within the regulated private sector is warranted" — a feeling shared with Senator Adlai Stevenson Jr who last week introduced a bill into the US Senate requiring mandatory registration of all appropriate experiments not currently covered by the guidelines.

Dr Bingham has suggested that the new panel should be established under the chairmanship of Dr David Rall, Director of the National Institute of Environmental Health Sciences — and that it should include not only members of the NIH's Recombinant DNA Advisory Committee, but also technical representatives from unions, management and public interest groups.

She also suggests that OSHA might review the NIH guidelines and publish them as interim guidelines for industry. "While these do not hold the force of regulations, they would indicate our support for control in this area and would also produce information to employees indicating what we feel are appropriate precautions and procedures of recombinant DNA use".

Dr Anthony Robbins, Director of the National Institute of Occupational Safety and Health (NIOSH) told *Nature* this week that he agreed with OSHA that the time was appropriate for detailed consideration of the implications of occupational exposures. Commenting on the fact that, in the past, OSHA has been reluctant to involve itself in the debate over the safety of occupational activities involving recom-

binant DNA techniques, Dr Robbins said that previously the agency had hoped this would be done elsewhere. "Since no one else seems to be doing this, it seems to be the time to move", he said.

NIOSH is, in particular, suggesting that a registry be set up of all workers involved in such activities to enable the long term health effects to be adequately monitored. And as a precedent, he points to the recent decision by the Swedish government to set up a commission on the occupational hazards chaired by the head of the National Labour Protection Board.

No response to Dr Bingham's proposals has yet been formally received from Dr Fredrickson, who last week issued virtually unchanged revisions to the NIH guidelines which he had proposed last November and which significantly reduce containment requirements for a wide range of experiments.

In particular, Dr Frederickson has implemented his proposals that most experiments involving the bacterium *Escherichia coli* K-12 be reduced to P1 plus EK1 containment levels, and that such experiments no longer need be registered with the NIH.

In announcing his decision, Dr Fredrickson said that he had received five letters of comment suggesting that the relaxation of the guideline was premature. However he had also received more than 170 letters giving general approval to the proposed changes, which he had decided to implement as originally suggested.

Dr Fredrickson's announcement, widely welcomed in the scientific community, coincided with the introduction of the bill tightening up the requirements for private industry introduced by Senator Stevenson, chairman of the Senate Subcommittee on Science and Space. Explaining the general purpose of his bill, Senator Stevenson said that in a time of general concern about the health of industrial innovation, the conditions surrounding recombinant DNA research seemed "unusually favourable" for the achievement of significant innovation — and that in the circumstances government regulations should not be undertaken lightly.

"However the modest registration requirement I propose is justified as a minimal precaution against inadvertent harm to public health or the environment. Far from impeding the orderly progress of the research and its commercial exploitation, mandatory registration may actually facilitate it." □

Earth sciences and remote sensing agreements signed with China

THE United States and the People's Republic of China last month signed two protocols for scientific and technological cooperation in earthquake studies and earth sciences, as well as a memorandum of understanding on the use of the Landsat earth resources monitoring satellite.

The signing of these accords came almost exactly a year after President Carter and Vice-Premier Deng Xiaoping signed a general agreement on scientific and technological cooperation between the two countries in Beijing (Peking).

The earthquake studies protocol includes cooperation in earthquake prediction, earthquake hazards evaluation and earthquake engineering, as well as other related basic and applied studies. Joint research in these areas is to begin immediately.

The other protocol covers research in areas such as marine resources, energy resources, marine geology and geotectonics. The two protocols cover various forms of cooperation between scientists in the two countries, including exchange visits, joint organisation of scientific conferences, symposia and lectures, and the exchange of specimens and standard samples.

Under a separate agreement with the National Aeronautics and Space Administration the People's Republic will purchase a ground station to receive, process, archive and disseminate earth resource data from the Landsat D satellite.

The station will be located near Beijing and the Chinese Academy of Sciences has agreed to help share in the cost of operating the satellite by paying an annual access fee of \$200,000, beginning six months after the station starts to receive data.

Other areas agreed by the Commission for possible future cooperation included aviation, statistics, nuclear energy (including nuclear physics and nuclear fusion), electronics and telecommunications. Administration officials in Washington last week rejected suggestions that moves to improve scientific and technological links with the People's Republic of China were directly related to the recent cooling of political and scientific relations with the Soviet Union.

Appearing before a Congressional committee hearing, Dr Frank Press, Director of the Office of Science and Technology Policy, said that scientific exchanges with China had been a positive element in the process of normalising US-China relations.

David Dickson

Andrei Sakharov

US cuts back on official exchanges with USSR

THE US scientific community has been almost unanimous in condemning recent moves by the government of the USSR, in the occupation of Afghanistan and the internal exile of physicist Andrei Sakharov.

But while many support the legitimacy of individual protest, there remains uncertainty over how far official reaction should undermine the network of bilateral exchange agreements which have, in recent years, begun to produce mutual benefits.

On the one hand, Congressman George Brown, last week introduced a bill calling for a one-year moratorium on US-Soviet exchange except those essential to national needs or a matter of extraordinary circumstance of individual conscience.

The Carter administration, however, while keen to make clear to the Soviet government that it is not 'business as usual' for scientific exchanges, is also concerned to maintain what it can of the existing framework for scientific cooperation.

Thus all high-level meetings between scientists and scientific administrators from the two sides are being deferred. Three such meetings have already been postponed since the occupation of Afghanistan, and all current exchange arrangements are undergoing close review.

But the administration is at present keeping the door open for low-level contacts between scientists which it considers to have a purely scientific or humanitarian purpose. For example, a scientific delegation from the USSR studying the biological control of pests has been allowed in — but a delegation coming from the USSR to discuss science policy has not, since it was lead by a deputy minister.

The administration's determination to keep open channels of communication,

even if limiting activities in these channels, was supported by Academy President Dr Philip Handler, who will be leading the US delegation to the scientific forum in Hamburg later this month to discuss the progress of the Helsinki accords of 1975. But he emphasised the Academy's position that the main responsibility lay on the shoulders of individual scientists.

Uncertainty over how tough the administration should be is reflected in the debate over what type of strategy the US delegation should pursue at the Hamburg science forum.

Few currently support a complete boycott of the meeting. And there seems general agreement that human rights considerations are central to international scientific communication and exchange.

But given agreement that the forum should discuss, in Dr Press's words, "the context within which scientific cooperation takes place, not simply those scientific subjects which are amply discussed in many other settings", opinions are divided on

what such discussions should aim.

Dr Paul J Flory, for example, Emeritus Professor of Chemistry at Stanford University and a member of the US delegation, suggested that the scientific community should "reshape its criteria for participation" and lay down a set of minimum conditions for future international collaboration.

These might include the condition that "negotiations should be in the hands of scientists, not governments", and that participants be selected "without regard for their political conformity, race or ethnic background".

Others have suggested more modest goals. In particular Dr John T Edsall of Harvard University, chairman of the American Association for the Advancement of Sciences' Committee on Scientific Freedom and Responsibility, has suggested that the forum should establish a working group to collect and review reports about obstacles to international scientific cooperation. **David Dickson**

Soviet Union responds to western reaction

WITHIN a few minutes of the news of Sakharov's exile reaching the West, the Royal Society sent a cautious telegram to the Soviet Academy of Sciences, deploring the action and asking for the Academy's comment. Six days later, the Academy made its opinion known in a formal statement by the Presidium.

The statement condemned the activities of Academician Sakharov as being directed "against the interests of our country and the Soviet people, actions that help to build up international tension and bring into disrepute the exalted title of Soviet scientist". Ironically, the Presidium's statement accuses Sakharov of "undermining" precisely those ideals of peace, arms limitation and detente that underlie his whole involvement with human rights. Last year, indeed, he spoke

out resolutely against overenthusiastic efforts to link US willingness to sign SALT-2 with human rights in the USSR.

Nevertheless, the statement contained no indications that moves would be made to expel Sakharov from the Academy. While feelings of solidarity may so far have prevented Sakharov's fellow-Academicians from expelling him from their ranks, there is little doubt that scientists of dissident outlook and lesser prestige now feel themselves under increasing threat. Andrei Tverdokhlebov, the young physicist, who had formerly worked closely with Sakharov in the human rights movement was expelled to the West just in time to testify before last week's congressional hearing in preparation for the Hamburg scientific forum. And a number of Jewish refusniks, notably Naum Meiman the mathematician and Aleksandr Lerner the cybernetician, have expressed fears for their own liberty.

Meanwhile, the Soviet authorities are attempting to counter western concern by denying that Sakharov has been, in any sense, exiled. "Administrative banishment", said one Tass statement "is neither 'arrest' nor 'exile'." Gor'kii, said comentator Yaroslav Khabarov on the foreign service of Moscow radio, "is one of the most beautiful towns in Russia, as well as a major industrial, cultural, and scientific centre with its own university". By simply stripping Sakharov of his awards and titles, and "moving him outside the city of Moscow", Khabarov explained, Sakharov has been given "the opportunity to continue to work in keeping with his profession". **Vera Rich**



Soviet Union

New appointment binds science closer to industry

ACADEMICIAN Gurii Marchuk has been appointed Chairman of the State Committee for Science and Technology, in place of Academician Vladimir Kirillin, whose resignation, was announced on the same day as the banishment of Andrei Sakharov from Moscow.

The appointment of Kirillin's successor came remarkably quickly. The post is of ministerial rank (and carries with it, virtually automatically, the position of a Deputy Chairman of the Council of Ministers). Posts of this calibre are seldom filled immediately — a lapse of several months has, of recent years, been the rule. Moreover, at 54, Marchuk is unusually young by Soviet standards, for high office. The average age of the country's leadership is in the late 60s.

Not that Marchuk is unknown to Soviet science. He has already held the corresponding position of head of the Commission for Science and Technology of the Soviet of Nationalities (lower house). More important, he is head of the Siberian Branch of the Academy of Sciences.

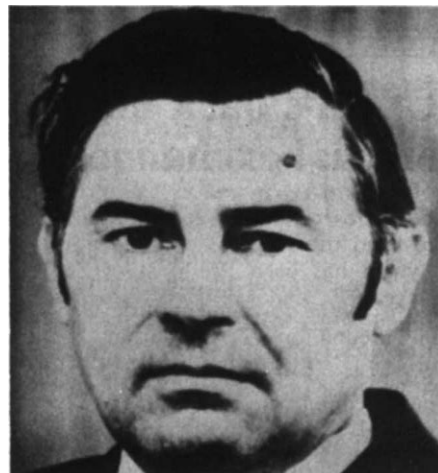
The Siberian Branch of the Academy was set up in 1957, housed in the new

Akademgorodok (Academic Village) at Novosibirsk, and briefed specifically to work on scientific problems of greatest relevance to the development of Siberia and the far east. By 1966, the branch had become surrounded by a network of research institutes, design bureaux and experimental production factories subordinated to individual ministries but under the scientific supervision of the branch.

This system of dual control has caused concern to a number of Soviet scientists, who have felt that the ministries might burden the R&D facilities of the branch with small-scale, day-to-day tasks, or even bypass the scientists altogether in decision-making.

Since he took over the chairmanship of the branch in 1975, Marchuk has written extensively on the dual control problem. Although at times he has complained that certain ministries have been lax in providing suitable conditions for successful work, his overall view is that the dual control system works well.

Of recent years, there has been constant emphasis in the Soviet press that science and industry must be more closely linked.



Marchuk: head of the Siberian Branch of the Academy of Sciences

Last November, when castigating the State Committee for slackness, Mr Brezhnev suggested that the Academy of Sciences should take a greater lead in organising the applied research the country needs. The precise division of labour and responsibility between the State Committee and the Academy is a complex subject (the head of the State Committee is regularly a Vice President of the Academy). Marchuk's Novosibirsk background, however, suggests that the long-discussed Soviet policy of integrated planning and the close coordination of research and production will be a keynote of the next five year plan for science. **Vera Rich**

United Kingdom

Industrialist made part-time director of biophysics unit

THE UK Medical Research Council (MRC) has taken the unprecedented step of appointing as a part-time director of one of their research units a scientist who is permanently employed within industry. As soon as Professor Maurice HF Wilkins retires as Honorary Director of the MRC Cell Biophysics Unit at Kings College, London, he will be replaced by the combination of Dr David Rees, 43 year old Executive for Scientific Policy for Unilever Research and 56 year old Professor Brian Boycott who is on the Unit's staff. Whereas Professor Boycott's post will be full time, Dr Rees will be in the service (and pay) of the MRC for only one day per week, on average, and will retain his present position with Unilever.

The hunt for a new director has been on for well over a year since Professor Wilkins let it be known that he would like to retire as soon as a replacement could be found. But it has proved extremely difficult, and ultimately impossible, to attract someone of the right calibre and scientific background to be director. The solution adopted is based on the belief that Dr Rees' scientific credentials as a

carbohydrate chemist with a strong interest in cell biology will complement those of Professor Boycott whose expertise is as a neuroanatomist of the retina.

Professor Boycott told *Nature* that he was glad to have someone familiar with current molecular biology as an Associate Director. The MRC expects Dr Rees "to inject good science into the unit" first by spending a continuous two to three months there and then by one day a week appearances and through having two of his research staff working in the unit. In return they hope that members of the unit will wish to sample the facilities and atmosphere of the Unilever Research laboratories.

A spokesperson for the MRC said that the link with industry was an experiment as much of serendipity as of design but that the MRC was now anxious to foster relationships between itself and industry. Although no other links were currently planned, as opportunities arose they would be considered seriously. □

Temperate vegetable seed bank set up

OXFAM, the Oxford-based famine relief organisation, has raised £700,000 by special appeal to establish a seed-bank for vegetables. A target of some 10,000 species

will be collected in a -20°C store associated with potting facilities, laboratories, and greenhouses.

According to Oxfam's director, Mr Brian Walker, two-thirds of the seeds collected will be temperate, and only one-third tropical; but, says Walker, "in many parts of the Third World people eat temperate vegetables: cauliflower, onions, carrots, potatoes, cucumber, tomatoes."

"In a tropical country you only have to go a little way into the hills to meet temperate conditions. A large part of India's potatoes are grown in the hills." Moreover, winter in the northern plains of India was little different from the growing season in the US.

The origins of the Oxfam initiative go back to the Rome food conference of 1974, when it gathered a group of senior scientific advisors to survey world seed-banks and report on deficiencies. The group reported that while there was good coverage of cereals, there was no embracing collection of vegetables, which are important particularly for people growing cash crops around the borders of towns.

The seed-bank will be established at the UK Agricultural Research Council's Vegetable Research Station at Wellesbourne, Warwickshire. Oxfam's £700,000 will pay the capital costs, and the running costs for the first seven years. Then the bank will be taken over by the ARC. □

NEWS IN BRIEF

Stanford president to head Rockefeller Foundation

Dr Richard W Lyman, currently President of Stanford University, California, has been chosen from a field of 1000 nominees to head the Rockefeller Foundation. Dr Lyman has been President of Stanford since 1970, and will leave his post in the summer. He will succeed Dr John H Knowles, who died last March, as President of the Foundation, of which he has been a trustee since 1976. University officials at Stanford will meet next month to discuss the selection of a new president.

US airlines told to restrict ozone intake

TEN years ago, it was a question of whether high flying aircraft would cause permanent damage to the Earth's ozone layer. Now US scientists are concerned that an excess of ozone inside such aircraft could be a hazard to passengers. And the US Federal Aviation Administration has issued rules requiring aircraft to reduce the amount of ozone entering aircraft, based on standards established by the Occupational Safety and Health Administration.

Initially proposed in 1978, the new rules will require airlines to screen out most ozone as it enters airline cabins by means of charcoal filters — or to follow routes that avoid areas of high ozone concentrations. On flights of less than four hours the maximum concentration of ozone will be 0.25 parts per million. On longer flights, the average level must be no more than 0.1 parts per million.

Pugwash takes up East-West dialogue

THE informal diplomacy practiced for 25 years by the Pugwash conferences was brought into play on 19-20 January in Geneva to "take up the slack caused by the present suspension of official East-West dialogues". Convened by the Pugwash Executive Committee, thirty three participants from 19 countries including high ranking Soviet and NATO officials met to discuss "the critical nature of current developments and the increasing danger of nuclear confrontation". Described as "useful", the talks included exchanges on the subjects of Afghanistan and the destabilising effects of the NATO nuclear modernisation programme. The participants will continue discussions at another specially convened meeting to be held in mid-April.

In the meantime the British Council of Churches is continuing its campaign against the installation of the latest Polaris missiles on UK submarines (29 November page 435). BCC representatives met with

the Ministry of Defence last week where they raised the question of the military relevance of a UK submarine nuclear threat that would be under NATO, not UK, control. Citing the opinions of Lords Mountbatten and Carver in support, BCC representatives cautioned the Ministry against renewing the Polaris as "a matter of political machismo".

PETRA to boost luminosity by ten

PETRA, the existing 19 GeV on 19 GeV electron storage ring at DESY, Hamburg, will install the last of 60 accelerating cavities in February and so for the first time reach its peak energy. And as the luminosity of storage rings increases rapidly with energy, it is hoped to raise the present 50 per day event rate by a factor of ten. This should make it possible at last to perform the 'forward-backward asymmetry' experiments which should detect the low energy 'tail' of the Z^0 , and bring early indications of the presence of the particle. According to the Weinberg/Salam unified theory of the weak and electromagnetic interactions, the Z^0 (intermediate vector boson) plays the same role in the weak interaction as the photon in the electromagnetic interaction. At 90 GeV, it would be the heaviest particle yet found.

Basques blow up nuclear power plant equipment

MEMBERS of the Basque separatist organisation ETA blew up electrical equipment scheduled for delivery to the troubled Lemoniz power plant near Bilbao last Sunday. A group of armed men captured the factory manager of the Segasa factory in Vittoria at his home, took him to the factory where they disarmed eight guards of their .38 calibre pistols and placed plastic explosive around special batteries intended for the Lemoniz plant. Warning the guards and manager to stay clear of the blast, the men left and the batteries exploded a few minutes later. The action occurred during a weekend of revolutionary violence in which ten people were killed in armed actions throughout the country.

Denmark shelves nuclear power indefinitely

THE Danish government last week announced an indefinite postponement of the introduction of nuclear energy. In a major policy shift, said to be strongly influenced by the accident at Three Mile Island, the ruling Social-Liberals supported by the Single Tax party, two smaller left wing parties and the major

trade unions — who had previously supported nuclear power — decided to defer any parliamentary action on the question.

Citing uncertainties about safety and waste storage, Energy Minister Poul Nielsen pointed out that Denmark was too small to support safely more than five nuclear plants which would only supply 15% of Denmark's energy needs. In its place, development of North Sea gas in conjunction with Norway is expected to cover the major part of Danish, Swedish and Norwegian energy needs well into the next century with enough left over to export to West Germany. The government will now draft a new energy plan that will emphasise, a shift from oil to gas, energy conservation and increased commitment to the development of alternative energy sources.

French researchers protest CNRS reorganisation

FRENCH scientists of the Centre National de Recherche Scientifique continued their opposition to management reorganisation plans by holding a one day strike and demonstration last week. More than 1500 research scientists marched through Paris to the offices of the Secretary of State for Research to protest against recent changes in the law which would introduce a new four year probationary period, lower the maximum age for entry to research to 27 years and permit forced mobility at the discretion of research directors. The trade unions representing the researchers sent an open letter to Prime Minister Raymond Barre signed by 1000 scientists including 300 research directors, senior researchers and professors that demanded a suspension of the decrees as measures that "would compromise gravely the future of basic research in France".

SRC clamps down on post-docs

THE UK Science Research Council has instructed its research directors to apply "rigidly" all rules concerning employment of post doctoral research fellows. The orders, contained in a memorandum circulated to SRC research establishments last November will effectively prevent research scientists on post doctoral contracts from gaining extensions or further contract work when their present contracts expire. The SRC defends its policy in ethical terms. Stating that the reasons for the policy were not financial — "very few people will be affected" — an SRC official said "we have a moral responsibility to encourage new ideas. People who have been here six years have had their chance and now they must make way for fresh blood".

FEATURES

Research centres, libraries, journals flourish in new China

In the second of three articles on the development of science and technology in China, **Tong B Tang**, research fellow at Darwin College, Cambridge, UK, who visited China at the end of last year, examines scientific services

MOST Chinese scientists — indeed, the people as a whole — are friendly, forthcoming and purposeful, yet modest and earnest (sometimes to the point that reserved westerners find uncomfortable to reciprocate). Their optimism is derived from the positive and negative lessons of the Cultural Revolution (reputedly assessed by Chairman Mao to be 70% and 30% respectively), open discussions on present problems, and from a common resolve to achieve the four modernisations in agriculture, industry, national defence, and science and technology. To this aim, the political decision-making system is being scrutinised and modified to prevent the recurrence of a dictatorship by a minority, like that of the Gang of Four (GoF).

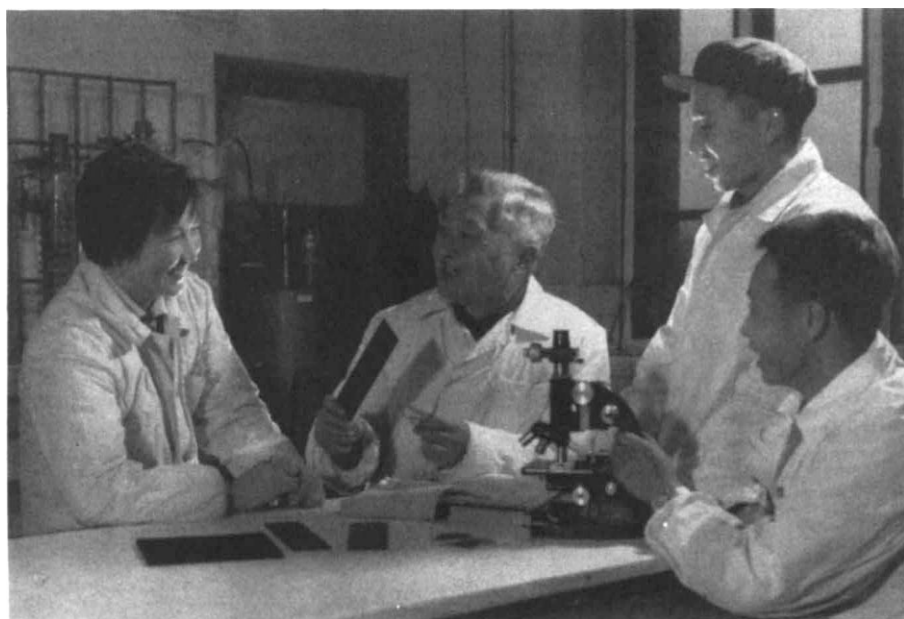
Both science and scientists suffered under the GoF. This was probably due not so much to deliberate policies as to the GoF's broadside attacks on "revisionism" and on people it labelled as "capitalist-roadsters". Although the GoF had some supporters its treachery became increasingly clear with time: its attacks

constituted the means rather than the end of its attempt to seize power. Sectarianism resulted and this reached a peak in 1975 when Mao condemned the factions semi-publicly. The aim of the present leadership is an extended period of stability and economic construction. The conditions are right for the growth of science.

Not surprisingly the Scientific and Technical Association, which barely existed three years ago, is expanding. Often affectionately referred to as the 'home' of scientists, it sponsored some 150 meetings in 1978; the number last year exceeded 650. The newest members include a futurology society and a solar energy society. As well as its function as a learned society, the association is responsible for the popularisation of science, which I shall talk about next week. Neither it nor any of its member societies acts as a qualifying body.

After the reopening of their 'home', our Chinese colleagues are also benefiting from other improvements. Problems had included insufficient time for research owing to a shortage of assistants and too many administrative commitments and

Researchers at the Institute of Applied Chemistry, Kirin, discussing the structure and performance of a new polymer



The Academia Sinica at Guangzhou (Canton)

family inconvenience such as wife and husband having to work in different institutions in different places. Now that research and/or teaching are judged to be the scientists' main duty, conditions of work are guaranteed. The buildings and appliances such as power points are often old and spartan by western standards, but functional. In general there are still not enough instruments yet, reassuringly, for the most part they are designed and produced in China. Laboratories may look run-down, but everyone using them knows exactly how the equipment works. Usually every bit of the equipment has not only been designed and built by the experimenters but is also maintained by them. Of course, there are some negative aspects. In a few places I noticed, for example, that resources appeared underused. But the main feeling is one of optimism.

Most Chinese scientists (some 25,000 out of 36,000 of those in the Academia's institutes) were trained after the Liberation and before the Cultural Revolution. They were educated by teachers produced by the old social system; through remoulding and re-education, however, they are judged to have become "part of the proletariat" now that the gap between manual and professional labour is considered overcome. Many scientists are commended

as advanced workers and are thus entitled to wage increments (the latest nationwide wage increase, on 1 November, was for 40% of state employees.) More important, more scientists are now allowed Party membership; they can be elected as deputies to the National People's Congress, or appointed to authoritative positions. In the past decade, a career structure, by qualification and seniority, has been designed and scientists can look forward to promotion.

A few institutions now adopt the convention that the authorship of papers will serve as a simple criterion of a member's contribution. All journals in China are, of course, published by the state and they have mandatory rankings, a paper in *Scientia Sinica* carrying the most weight. This may encourage the formation of self-centred sub-groups each pursuing prestigious research; the decline of collective authorship in the journals could be a reflection of this. More inventively, some institutions pursue the practice of arranging exhibitions of research results. All members are invited to assign 'work points' to the research on display according to personal judgements of its quality. Promotions are decided by the institution's 'academic committee' which refers to these collective evaluations. Some institutes also organise symposia at which their research is described and then discussed by co-workers invited from other institutes or universities.

Since 1978, scientists have been paid by the state for papers published whereas in the previous decade not even authors of books received royalties. Much stress is being laid on information science. Last August the China Society of Libraries was formed. Some of the larger university libraries plan to be computerised within a few years. Also in 1978 the Scientific and Technological Literature Press began issuing, for a whole range of subjects, monthly and bimonthly indices of foreign papers and specifications of open patents being applied for in Japan.

A national shortage of wood and wood pulp has constrained the publication of books and periodicals but is slowly being overcome. China now publishes a total of 1,200 periodicals in all areas and more will probably be launched. In electronics and communications technology, for example, there are some 20 primary journals with a general circulation. I also saw the following titles which are exclusively devoted to classical disciplines within physics: *Acta Physica Sinica*, *Wuli* (physics), *Acta Mechanica Sinica*, *Mechanics & Practice*, *Acta Acustica*, *Luminescence in Solids & its Application*, *Laser Journal*, *Gaofengzi Wuli* (high polymer physics), *Diwen Wuli* (low temperature physics), and *Physica Energiae Fortis & Physica Nuclearis*.

Foreign journals are also used. Because of their high costs, however, most of them are centrally subscribed by airmail and then reprinted, the home-produced versions



Students at the University of Science and Engineering, Tsinghua, engaged in an electronics experiment

then being nationally distributed. The copies usually manage to reach the library shelves with no more than a few months' delay. There are no cover-to-cover translations into Chinese, and some readers may find this a problem.

The language problem can also affect visiting scholars. Speakers may find that their talks and the subsequent questions and answers are translated verbatim, sentence by sentence, with the help of the entire audience. Despite such difficulties, it is certainly a positive development that visits, in either direction, are increasing in both frequency and in the variety of disciplines covered.

'Centres of Scientific and Technical Exchange with Foreign Countries' have been set up in Beijing, Shenyang, Shanghai and Guangzhou (Canton) to assist the Foreign Affairs Bureau of the Academia Sinica and the Ministry of Education with scientific exchanges. The Scientific and Technical Association also oversees exchanges. Together with the US Committee on Scholarly Communication with the People's Republic of China (PRC), it is arranging for senior scientists as well as graduate students to stay for extended periods in China and participate in joint programmes. Sino-British cooperation in education, public health and culture was sealed by a five-year agreement signed at 10 Downing Street on 1 November 1979 during Hua's visit.

China now hosts international conferences more often, examples being the International Rice Research Workshop at the end of October last year in Guangzhou, and one on particle physics one month ago, at the same venue, under the chairmanship of Qian Sanqiang.

More than 1,600 academics, 180 research students and 420 undergraduates have been sent by China in the past two

years to the US (500), UK (300), West Germany (200), France (200) and Japan (100). The overwhelming majority (1,800) are studying natural sciences. Advanced crash courses in languages, more often needed than not, are given just before departure or immediately after arrival at the host country.

Language will not, of course, be a problem for visitors of ethnic Chinese origin if they can speak the standardised dialect. Some of them have established close contact, and are on the 'academic committees' of some institutions. Scientists such as Chen-Ning Yang, Nobel prizewinner for the discovery of the violation of parity conservation, have made particularly valuable contributions, by participating in or advising on research, and inspiring young people, as well as cultivating friendship between China and their adopted countries.

'Sister universities' constitute another novel form of overseas ties. Zinghua at Beijing is affiliated to the University of California at Berkeley, Zhongshan in Guangzhou to the University of California at Los Angeles, and Nanjing to the University of Wisconsin. China is taking part in more bilateral and international scientific programmes. Recent examples are the measurement of $\nu_e e$ interaction cross-sections at the Fermi Laboratory and the Global Atmospheric Research Programme sponsored by the World Meteorological Organisation. As a result of these overseas links, increasing attendance at international meetings, and of submission's to western journals by Chinese scientists, it is now easy to find out who's doing what in the country. One source of information is the *Directory of Scientific Research Institutes in PRC*, produced by the National Council for US-China Trade in Washington, D.C. □

One man, two cultures, one discipline

Robert Walgate speaks to a former film-maker who subjected himself to the 'discipline' of science, and succeeded

"IT WAS a pretty nasty business" said Michael Shallis, gesticulating from his office chair against a blackboard backdrop of equations and graphs, "making cinema commercials. I spent a year as a staff film director for the Rank Organisation." He laughed. "It was really quite ludicrous — we'd make about six a day."

Dr Shallis is now a research fellow with Professor Donald Blackwell in the Department of Astrophysics at Oxford, making ultraprecise, painstaking measurements of the oscillator strengths of spectral lines of iron and other elements, to create a new basis for classical stellar spectroscopy.

He took no interest in science at school; avoided university; and joined a film school in London. He became an editor with Movietone News, the last of the cinema newsreel makers.

But during the sixties he became "exposed to science in one or two curious ways. I was absolutely spellbound by a series on television by J. Z. Young, the biologist — I've still got the little BBC two and sixpenny booklet that went with it. I found that the way he thought was very like the way I thought".

"In the film industry, the way I thought was very different from the people I was working with. I can't really explain it because I didn't at the time understand it myself."

Could he understand it retrospectively? Very diffidently Shallis said that he had an objective curiosity which he could see reflected in Young; whereas the people in the film business work mainly from their emotional rather than intellectual centres.

Shallis felt he wanted to make a film about the way science shaped us, how society might develop — but he found it difficult to interest a backer. "I spent quite a lot of time seeing if I could combine some sort of interest in the sciences with other things . . ."

Shallis's shift to the physically objective, the rational — wasn't that totally against the trend of the times, which was to abandon science and technology for the mystical?

"Yes, as that happened I did exactly the opposite. Then I reached a decision in

1970, when I was 27, that the only way I'd understand about science was by going and doing it."

The next thing was to work out how to do it, with A-levels in art and English. He started reading books on science, and began to see that he was more interested in cosmological and astronomical ideas than biological. Shallis was amazed to discover Professor Elton's conversion course from arts to sciences at the University of Surrey. He spent a year on that and emerged four years later with a first in physical sciences. Both courses have now closed.

There was a lot of spectroscopy in the course; he was interested in astronomy (he built a photometer for his telescope as a final year project); and when it came to choosing where to do research he chose Blackwell's group.

"I don't regret the decision. I think the work we do here is the most interesting in ground-based astronomy in this country. I'm a pragmatic person; so I'd always have been an experimenter or an observer. I was pragmatic in film-making too, interested in behaviour rather than inner psychological motives."

Wasn't the kind of science he was doing with Blackwell very painstaking and unspectacular? Wasn't the amount of emotional return that he could get from measuring the width of a spectral line pretty small?

"Well it was something I really needed to do. From the almost 'anything goes' attitude within films, to the highly disciplined work that we do . . . I had to work very slowly and carefully, to learn that sort of rigorous discipline, which I'd never learnt in the arts at all. So I needed in the sciences very much to discover that rigour — in order, in fact, to release my creativity."

"The gap between the two cultures is probably widening. Up until the age of 27 I'd never met a scientist. At school they were completely a different breed. But now I've crossed the gap and discovered they are not a different breed at all, but actually doing the same thing. That has always interested me."

So why is the gap there? Partly for social and educational reasons — it's the way we

are brought up; partly because scientists like it to be there. There's a sort of class distinction on both sides.

But also it's a matter of access; anyone can buy a book or a record. "You can go up to a man in the street and say have you seen the latest Woody Allen or have you heard about the Picasso exhibition at the Tate gallery and he'll say yes I have, I haven't seen it, but I know about it and I think Picasso's bloody awful; but if you ask him about the latest experiment in particle physics he'll look at you and gape and say, Ugh! At least the creative arts are accessible."

"People's hostility to science is growing and will continue to grow. As pollution and nuclear accidents and these things continue, hostility will grow and will grow rampantly."

"One of the problems scientists face is that they are so bound up in what they are doing, they generally fail to see why other people aren't. They find it very hard to see that other people don't necessarily find what they are doing fascinating. One sees this in the arts too. But it's more important in the sciences, because of the impact of technology on lives."

Now that Shallis has been through his creative period and his discipline he wants to loosen the discipline again.

"In a sense I want to integrate my experience. There are a lot of things I want to say about it. Which is why I want to move more into communication, writing books, teaching with the Department of External Studies. However fascinating research is, I still feel it is missing out on a lot of things, and there are other things I want to do."

"There are a lot of gaps to be filled in popularisation. The man in the street has very lopsided and wrong views about the nature of science, in ways that affect his life — like interpreting statistics."

"The thing that concerns me most within science itself is — I'm stepping carefully here — the lack of an ethical basis and the lack of a spiritual basis. It worries me very greatly."

"I came across a quotation from Jung last night: 'The rationalist unbeliever who has no gods left in his heart is tormented by the devils in his brain'. I think that's very good. Science as a collective thing has become a sort of rationalist unbelieving



Michael Shallis: film-maker turned astronomer

entity with no gods left in its heart. And so it is tormented by the devils in its brain.

"One sees it in individual scientists to a greater or lesser extent. There's a scientist I know who believes — and it's a very widely held view — that ultimately everything in the universe can be reduced to physics. It is a very very sterile view, and one that I think is untenable. And in a sense their lives reflect this, their lives become extremely barren. Science is getting more and more materialist.

"Dora Russell said in her autobiography that science became inaccessible with relativity theory; and she saw that as the source of a lot of difficulties in the 20th century. Relativity theory was the first step towards obscurity."

How could ethics be grafted onto or grow out of science? "I'm sure there can be an ethical basis for science. The ethics have to come into the applications and the choice of science. It's tied up with the ethical basis of society."

Doesn't the scientist now have the ethics of the mercenary? Will he not do his science for whoever pays? "Yes. He thinks the freedom to investigate nature how he wants is his ultimate right. But I don't think anyone has ultimate rights.

"Take the use of animals. What ethical basis can you have for doing experiments with animals? The scientific community says animals should be available for whatever purposes we deem we need them for. We will be as humane as we can within those terms, but ultimately our interests over-ride the concern for the animals. I think that is a devastating ethic for science."

The discussions are always expedient, rather than ethical. "Of course that is a difficulty that our society in general faces — society doesn't have an ethic any longer that gives us any basis for our political judgements, our economic judgements, our personal judgements."

What about the spiritual element? "It is certainly lacking. Very few scientists have a feeling of awe about the universe; a few talk about it; but the whole thing becomes rather mundane for them."

But what should a group do? Every Saturday morning gather round and have a contemplation exercise? Shallis, laughing: "Maybe they should, quite possibly . . . I haven't any answers, you see, I'm just beginning to become concerned with these things, and the more I think about it the more I become worried."

Does Shallis get any spiritual return from his spectral lines? "Out of science I get very little." But what kicks, I asked, *does* he get?

He is very enthused by the programme of Professor Balckwell's department. "Yes, that is very interesting, this is the kick, but so what? Can we justify that kick on other grounds? Is it enough? Matisse gets a kick out of doing a painting; a scientist out of his piece of science. On an individual basis that's fine. But what happens then?

Animal or vegetable?

VEGETARIANISM is a fascinating and delicate subject. It must be treated with tact because to many people it is of religious significance, but its scientific side compels interest and discussion. Bernard Shaw used to tell hostesses who invited him to dinner that he would not come to eat dead animals. He also refused to eat asparagus, which he said was a very nasty vegetable. His ebullient contemporary, GK Chesterton, in contrast, wrote some rollicking verses that made fun of non-meat-eaters, and non-milk-drinkers ("I will stick to wine and sherry, because they are so very, so very, very, very vegetarian").

An important step towards asserting the rights of plants was taken when it was found that cytochrome *c* of wheat and vertebrate animals had amino acid sequences so similar as to betoken the sharing of a common ancestor¹. Indeed, the sequences of the cytochromes *c* show a phylogenetic relationship throughout the eukaryotes and extending to the purple bacteria. We cannot even eat a mushroom without devouring a relative. Some people talk and play music to their house plants, and we may soon hear of a Society For The Prevention Of Cruelty to Vegetables.

Deficiencies in foods of vegetable origin have been responsible in many countries for slow growth in children and, in consequence, for undersized adults. Even in the "developed" countries, it is sometimes ruefully pointed out that farm animals often receive better dietary treatment than human beings. This may be partly because cows do not read.

About 40 years ago it was evident that something in meat and fish, an "animal protein factor", was needed by chickens and pigs to correct a deficiency in diets consisting of foods of plant origin. Soybean meal, although a valuable food, lacked the missing substance. It was amusing and paradoxical that a vegetarian friend of mine, Lester Smith, successfully hunted down the missing substance in beef liver. In 1948, he isolated the anti-pernicious-anemia factor, which was also the animal protein factor. Simultaneously, the substance had been isolated from fermentation of a soil microorganism *Streptomyces griseus*². The compound was re-named "vitamin B₁₂", and the catchy designation did wonders for it. US professional football players, so I read recently, sometimes receive vitamin B₁₂ injections to prepare them for the fray. Faith can move mountains.

Green plants do not make or need

Science has, where art hasn't, an impact on the world. In science we have a whole group of people doing it for that kick, but we have also a vast power structure influencing the lives of all of us; and it is out of overall control. It is enough to Matisse; whether



THOMAS.H.JUKES

vitamin B₁₂. It all comes from microorganisms. Animals receive their supply from milk, or by predation, or from bacteria in the digestive tract, or by voluntary or involuntary coprophagy. The ruminating cow has an internal fermentation vat from which vitamin B₁₂ gets in her milk to the benefit of lacto-vegetarians and other consumers of dairy products. Rabbits, less fastidious, consume their own faeces at night to obtain vitamin B₁₂. McGee³ described the preservation, drying and consumption of faeces by the Seri Indians of Tiburon Island in the Gulf of California, who felt that endurance for the hard warpath on prolonged chase was augmented by this practice. Perhaps this was another case of faith moving mountains.

The consumption of meat is currently under criticism because its production is wasteful of food resources, such as cereal grains, that could be consumed directly by people. Against this, it is argued that grains are deficient in iron, and are poor sources of proteins. It is also true that ruminant animals supply us with meat and milk while consuming large quantities of forage and roughage, such as stalks and straw, that are not edible by human beings. The fermentation vat of the rumen converts the roughage to soluble nutrients. Some of the rumen organisms, *Methanobacteria* are strict anaerobes that may be "living fossils" of a very ancient era.

(1) Stevens, F.C., Glazer, A.N., and Smith, E.L. *J. Biol. Chem.* 242: 2764-2779, 1967.

(2) Rickes, E.L. *et al Science*, 108: 634-636, 1948.

(3) McGee, W.J., *17th Annual Report, Bureau of American Ethnology*, Part 1, p.212, Washington, D.C., 1898.

someone buys the painting or not is an irrelevancy to him; but the products of science are used and manipulated. I have no objection to the individual scientist; it is the influence of the collective. But then where does one draw the line?" □

CORRESPONDENCE

Future world energy needs

SIR, — It would have been most instructive if we had been told the composition of the "Conservation Commission" of the World Energy Conference, whose 1978 Report on Energy Requirements between 1985 and 2020 was reviewed in *Nature* (November 15, page 344). Neither the Commission nor your reviewer appear to have addressed themselves to the possibility of meeting their projected goals. To reach them the Commission says that nuclear generating capacity must be doubled every six years from 1985. If we suppose that there would be no more than 200 1000 MWe reactors operating by that date, this growth rate would mean that 12,800 reactors of the same size would have to be built by 2020. To reach this number the world would have to build a reactor a day from 1985 onwards. Furthermore, since reactors are unlikely to last more than about 35 years, by 2020, this rate of building would have to increase to two per day unless of course demand miraculously levelled off at this point in which case it would merely be necessary to go on building a reactor a day for evermore.

The 12,800 reactors to be completed by 2020 AD would, during their lifetimes, need to consume up to 140 million tonnes of uranium metal, which is 10-15 times the 1979 estimates for the world's stock of uranium costing less than \$30 a pound to extract.

The UK government has recently given an estimate of £8 × 10⁸ for building a 1000 MWe nuclear plant. 12,800 reactors of this type would therefore cost a total in the region of £10¹³ in 1979 values. This and the fact that at present in the US it takes about 10 years between design and commissioning of a reactor (and reactors become steadily more complex in an effort to meet ever-stricter safety requirements) appear to indicate that the world's attitude to energy consumption will have to change radically within a few years from now.

In assessing energy "demands" one must remember that a 1974 UN forecast was that average energy "demand" in the developed countries up to 2020 AD would rise thirteen times as fast as population, whereas it was expected to fall in the developing ones. Projections of energy demand are therefore merely estimates of how much energy people will be willing and able to buy: they have nothing to do with opinions about what distribution of energy consumption would be socially or morally desirable. Perhaps we should start asking ourselves such questions now?

Yours faithfully,

R. B. TEMPLE

Physical Chemistry Department, University of Sydney, Australia

Biotechnology report

SIR, — I should be grateful if I might make it clear to your readers that the Biotechnology Report partly published by you (24 January, page 324) was not "made available to *Nature*" by the Working Party, who were unaware that you had obtained an early draft until two days before its publication. It was an uncorrected, tentative draft, sent to our three sponsors (ACARD, ABRC, and the Royal Society), and to many of those who gave evidence to us, in November. Their advice led to significant alterations, only completed on 21 January. What I hope will be the final draft has now been sent to the sponsors for approval of publication.

Yours faithfully,

ALFRED SPINKS

Wilmslow, Cheshire, UK

Short-term research contracts

SIR, — It is indeed heartening to read your valuable and informed coverage of the problems of experienced and accomplished scientists stranded and unemployed in their thirties and forties. It is equally disheartening that *Nature* is almost completely alone, both in the specialist and the national press, in drawing attention to the problem and its magnitude.

Why is everyone apparently so complacent? Are research directors and professors alike so committed to the exploitation of cheap and expendable labour that they see no need to speak out against a system whose iniquities they have been fortunate enough to escape and whose benefits they shamelessly enjoy? Why do we hear nothing from the heads of research councils and grant-giving agencies? It may be argued that the present situation has developed of its own accord. Does this mean that nobody should now begin to set matters right? Where does responsibility for perpetuation lie?

I issue a challenge, to the head of any research council or funding organisation, to defend the present system of short-term contracts in scientific research. I challenge him to defend the practice of signing away rights in common law as a prerequisite for the acceptance of such contracts. I challenge him to justify the expenditure of public money on the training and usage of men and women drawn from the most talented sections of the community, to be tossed into the dole queue before their full potential is realised.

Yours faithfully,

ROBERT JONES

Am Steinberg 67, Heidelberg, Germany

Pheasants and spies

SIR, — Having just returned from Kathmandu, Nepal, where a highly successful International Pheasant Symposium was held from 21-24 November, I was horrified to read your story headline 'India's wildlife projects under spy cloud' (29 November, page 435). In this story, under a cartoon depicting Kashmir pheasant beaters, you report that "Foreign collaborators might use the [World Pheasant Association's] pheasant project [for the re-introduction of the Cheer pheasant] as a camouflage for spying in the sensitive borderland".

Had you checked with the World Pheasant Association you would have discovered that the "foreign collaborators" for this project confine themselves to despatching batches of Cheer pheasant eggs from Heathrow to Delhi, where they are met by Indians, hatched by Indians and reared by Indians with the full knowledge and support of the state governments concerned; clearly they do not share your correspondent's fear.

To set up the project I have myself visited the Wildlife Departments and other officials in Lucknow (Uttar Pradesh), Simla (Himachal Pradesh) and Srinagar (Kashmir), all leading tourist centres and none of them on the 'sensitive borderland'. The publication of unmitigated nonsense like this can do the work of recognised charitable conservation organisations like the World Pheasant Association and its objectives nothing but harm.

Finally, the World Pheasant Association received from the Chief Minister of Jammu and Kashmir an invitation to hold the Second International Pheasant Symposium in Kashmir, (an invitation that is being accepted). Hardly the action of a government concerned with spies infiltrating its territory.

Yours faithfully,

K.C.R. HOWMAN

World Pheasant Association, Suffolk, UK

Standardisation of research citations

SIR, — Three groups of life science editors have tried to achieve the standardisation of reference citation for which P.C. Reid argues so cogently (17 January, page 242).

First, in 1973 the biochemists (IUB-CBJ)¹ published a reference list style suitable for journals adopting either the 'Harvard' system (names and dates) or numbers for citations in the text:

Abel, B.C., Cain, A.D., Adam, E. & Eve, A. (1976) *Tree J.*, 12, 90-99

Second in 1977 ELSE (European Life Science Editors' Association) and the Ciba Foundation organised a workshop on references and produced a set of suggestions² for a style that is also suitable for either names/dates or numbers in the text:

Abel BC, Cain AD, Adam E, Eve A 1976
Bark thickness in apple trees. *Tree Journal* 12:90-99 (or *Tree J* 12:90-99)

Third in 1978 the International Steering Committee of Medical Editors proposed what is now known as the Vancouver style³, suitable only for citation by numbers:

Abel BC, Cain AD, Adam E, Eve A. Bark thickness in apple trees. *Tree J* 1976;12:90-99.

The main differences between the first and second systems lie in the punctuation, the typefaces, and whether article titles are included or omitted. The main differences between the second and third systems lie in the way references are cited in the text and in the punctuation and position of the date in the reference list. All three systems recommend that the 'International List' (ISO 833-1974; BS 4148 Part 1; 1970 and Part 2:1975) should be used for abbreviating journal titles, in preference to the World List.

The ELSE-Ciba Foundation system suggests that journal titles might be either unabbreviated or abbreviated, provided that each reference list is self-consistent on this point. A later suggestion is that editors and publishers should allow both names/dates and numbers in the same journal or book, provided that each article or chapter follows one style consistently. Since the American National Standard (ANSI Z39.29-1977) and the draft International Standard (DIS 690) on bibliographic references both permit flexibility in the position of the various elements, including the date, in reference lists it would be a great step forward if the third system became more flexible on this, so allowing names/dates to be used in the text. The second and third systems would then be even more alike than they already are, and the work of authors, typists and typesetters would be enormously simplified. (Copies of the ELSE-Ciba Foundation system are available in either a full or an abbreviated form from the editorial department of the Ciba Foundation, 41 Portland Place, London W1N 4BN, to anyone enclosing a self-addressed adhesive label with their request.)

Yours faithfully,

MAEVE O'CONNOR

The Ciba Foundation, London, UK

1. IUB-CBJ. *Eur. J. Biochem.* 37, 201-202 (1973). (And elsewhere.)
2. O'Connor, M. *Editing Scientific Books and Journals*, p 176-185 (Pitman Medical, Tunbridge Wells, 1978). (And elsewhere.)
3. International Steering Committee of Medical Editors. *Br. med. J.* 1, 532-535 (1979). (And elsewhere.)

NEWS AND VIEWS

Communication between cells

from W.H. Evans

ANIMAL cells communicate in a variety of ways. The most direct and intimate communication must occur between assemblies of cells, allowing them to summate and coordinate their growth, development, division, metabolism, response to external stimuli and so forth. Exactly how this occurs is still a mystery, but as Unwin and Zampighi show on page 545 of this issue of *Nature* answers are beginning to be found at the cell's surface membrane — the repository of so many regulatory mechanisms.

A simple fact, appreciated widely only in the past 30 years — so persistent has been Schleiden's cell theory of 1838 emphasising that cells are self-contained independent units — is that most animal cells contain at areas of surface contact hydrophilic channels bridging the intercellular gap and directly connecting the cells' cytoplasmic milieu. These intercellular communicating channels can control the extent of equilibration of the cytoplasmic components, and they also provide the potential for the transfer or exchange of informational molecules across large sheets of cells — a property appreciated by developmental biologists. Provided that channel porosity is regulated, macromolecular individuality of the cooperating cells is maintained while the sharing of simple ions and molecules is permitted. The arrangement can appear as a sort of compromise between the independence seen in circulating blood cells and in nerve cells (which have developed alternative methods of communication using synapses) and the wholesale fusion of cells to form functional syncytia as seen in skeletal muscle.

By the 1960s a number of diverse biological observations converged to implicate plasma membrane junctions as the probable pathway for the rapid spread across tissues of injected fluorescent dyes, the passage of electrical current across heart tissue, and even the exchange of metabolic intermediates between cells in culture. Indeed, the last, termed metabolic cooperation (Pitts & Sims *Expl Cell Res* 104, 153; 1977), has provided a most powerful experimental tool to investigate the specificity requirements leading to exchange of metabolites and has shown that channels are formed rapidly — within 30 min of intercellular contact (Epstein & Gilula *J. Cell Biol.* 75, 169; 1977).

In 1966, Lowenstein (see *Biochim. biophys. Acta* 560, 1; 1979) proposed a model for a direct cell-cell pathway that could permit ions and hydrophilic molecules of up to ~1000 molecular weight to move more or less freely between cells. Soon after, the responsible anatomical structure in the plasma membrane of epithelial cells became clearer when Karnovsky and Revel used the lanthanum impregnation technique to distinguish clearly between tight and gap junctions between cells. The former seal off the intercellular space, whereas the latter have a 20–40 Å gap permeable to lanthanum. The use of the term 'gap junction' is perhaps unfortunate, for the intercellular gap revealed by electron-lucent tracers is bridged by leak-proof channels connecting the cell interiors. The functional distinction between these often-confused cell junctions could not be greater. Fast on the heels of the identification of gap junctions in tissue thin sections was the demonstration, by freeze fracturing the component plasma membranes, that gap junctions are easily identified as plaques of closely aggregated intramembranous particles. This quickly led to gap junctions being recognised throughout phylogeny — from simple coelenterates, such as *Hydra* to man — providing compelling evolutionary evidence for their functional importance.

The lattice-like nature of gap junctions is conspicuous when the junctions, purified from tissues, are viewed in the electron microscope after negative staining. Their striking hexagonal packing makes them attractive candidates for ultrastructural analysis by non-destructive electron microscopy and image reconstruction — approaches which have been developed by the Cambridge group using bacterial purple membranes. High resolution electron microscopy and X-ray diffraction have already provided plausible models of hepatic gap junctions in which proteins of the apposed plasma membranes are arranged hexagonally producing a channel traversing the membrane (see Bennett & Goodenough, *Neurosci. Res. Progr. Bull.* 16, 375; 1978). In this issue Unwin and Zampighi put forward a new refined model to explain junctional communication between cells across hepatocyte gap junctions, the largest biomembrane channels known.

By tilting negatively-stained junctions in the electron microscope, and analysing the pictures using Fourier and image

reconstruction techniques, the communicating unit (connexon) emerges as an hexameric polymer of six subunits spanning the membrane and protruding from either side. The subunits are tangentially inclined which explains the annular appearance of each connexon. The channel is approximately 20 Å wide at its exposed region, although the channel dimensions deeper inside the 'tunnel' are not resolved. Models of gap junctions should explain how channel porosity can be regulated between open (cells are coupled) and closed (cells are uncoupled) configurations, an important physiological function sensitive to changes in Ca^{2+} concentrations and/or pH. Unwin and Zampighi's model provides a neat and simple explanation for changes in channel porosity on the basis of maps of two junctional states by postulating a rotational displacement of the hexameric subunits, so opening and closing the narrow channel.

Models of junctions between animal cells have leapt ahead of our knowledge of junctional biochemistry and the nature of ions and molecules transferred in specific functional states. Crystalline lattice structures are often the outcome of molecular parsimony, and although it is often assumed that the hepatic gap junction, like the purple membrane, is composed of a single monomeric polypeptide, the biochemical evidence is still unsatisfactory. The lipid composition is unexceptional (Hertzberg & Gilula *J. biol. Chem.*, 254, 2138; 1979), and the isolated junctions, prepared by detergent extraction of plasma membranes, are strictly lipid depleted, detergent-binding fragments.

Some clues have recently emerged implicating cyclic nucleotides as possible important metabolic control signals transferred across junctions. It was shown by Lawrence *et al.* (*Nature* 272, 501; 1978) that hormonal stimulation can be transmitted between cocultured granulosa and myocardial cells. The cells were joined by gap junctions and since hormonal stimulation of both cell types is cyclic AMP dependent, it is possible that cyclic AMP is the agent responsible for cross-stimulation. Cyclic nucleotides may also feature in intercellular communication in heart tissue where synchronously contracting cardiocytes are joined by intercalated disks which incorporate gap junctions. The possibility arises that cyclic nucleotides first arose primordially as

signal molecules sufficiently tiny to move directly and rapidly across junctions between cells before they also became implicated in hormone receptor-mediated communication between organs. In the same vein are reports of a correlation between gap junction formation and hormonal activation in myometrium (Garfield *et al. Science* **198**, 958; 1977) and oocytes (Browne *et al. Science* **203**, 182; 1979). And finally, to the transmission of

hormonal stimuli between cells connected by gap junctions one can also add that the action of interferon is also likely to be mediated and amplified by the same intercellular pathway (Blalock & Stanton *Nature* **283**, 406; 1980). Clearly, now that gap junctions have emerged as widely-distributed plasma membrane organelles tailored for intercellular communication, the finer detail of their biological role still remains a wide-open question. □

Information exchange in the Palaeolithic

from Clive Gamble

DEVELOPMENT in palaeolithic culture can no longer be adequately explained in terms of increasing intelligence, for recent studies have demonstrated the existence, some 300,000 years ago, of mental ability equivalent to that of modern man. The dramatic developments in material culture, such as the appearance of art, now seem to be more closely related to changes in the amount and kind of information needed by palaeolithic societies, rather than dependent on the evolution of the brain.

Archaeologists have generally concentrated on the obvious in their studies of the earliest art. Understandably they have felt most at home when discussing the representational images that were either painted, sculpted or engraved by palaeolithic man. The renderings of animals and humans have been analysed in terms of composition and the chronological development of artistic styles. Their visual content has been interpreted in terms of either magical or religious significance. These images are seen as a key with which the archaeologist can unlock the psychology of early man while also providing an opportunity to investigate the cognitive development of the genus *Homo*.

These painted bison and carved figurines are however only part of the artistic output of palaeolithic man. A large body of less obvious art has also been recovered and consists of scratches on ornaments, pieces of bone, stones, the walls of caves and impressions drawn in clay. Their interpretation has barely proceeded beyond regarding them as the first 'doodles'; an analysis that stems from their apparently random execution. In a series of recent papers Alexander Marshack has subjected these scratches to a detailed systematic analysis (*Curr. Anth.* **20**, 271; 1979; *Form in Indigenous Art* (ed. Ucko) Australian Institute of Aboriginal Studies 1977). His method of investigation involves microscopic examination of how these abstract images were made. His studies

have revealed that far from being random these scratches were formed in an intentional sequence whereby composite abstract images were built up. He has further grouped the scratches on the basis of repeated motifs into descriptive classes such as meanders, fishlike images, zig-zag and parallel lines.

The majority of these scratched signs and their painted counterparts on cave walls are found associated with upper palaeolithic stone tool assemblages. They appear some 32,000 years ago at a time when the fossil record indicates the replacement of *Homo sapiens neanderthalensis* by *Homo sapiens sapiens*. This is also the period when the first representational art is found in Europe. However Marshack has also been able to show that in two instances the same intentionality in scratching signs predates the appearance of modern man. This important finding suggests that the potential for image composition of this kind was possessed by both neanderthal and *Homo erectus* populations. This raises the possibility that the traditional explanation for such behaviour, which regards art as a unique developed mental ability of *Homo sapiens sapiens*, is no longer entirely adequate. Wynn (*Man* **14**, 371; 1979) has analysed the operational intelligence of handaxe manufacturers from Isimila, a site comparable in age to Pech de l'Azé where Marshack has documented the first intentional scratched meander. His analysis uses Piagetian genetic epistemology as a framework to investigate the cognitive procedures involved in the manufacture of these artefacts. On the basis of this study Wynn concludes that the hominids who made the handaxes were not significantly less intelligent than any modern adult. His important conclusion is therefore that increasing intelligence cannot be used as an explanation for developments in material culture over the past 300,000 years.

These provocative studies challenge what has to be explained by palaeolithic archaeologists. If we accept their

conclusions then it becomes clear that it is no longer sufficient to look at human evolution over the past 300,000 years as simply a process of increasing intelligence and offer explanation in terms of changes in the brain. Instead we must approach the problem from a different perspective and look for changes in social circumstances which selected for and brought into play these latent patterns of behaviour.

One current line of enquiry stresses the role of information exchanges in palaeolithic society. In this context art and the scratched signs become part of a visual system of communication designed to handle information in this mode. The sudden appearance of art in the upper palaeolithic is thus a response to handling more and different types of information. Style provides the means by which order is brought to this form of communication so that messages can be sent and received. All items of material culture can be modified by stylistic rules to provide such additional visual information. This perhaps explains why there is such a sudden increase in stylistic behaviour among the stone tool assemblages at the same time as the art first appears since, as Wobst (*Papers for the Director* (ed. Cleland) Anthropology Papers, **61**, Museum of Anthropology, University of Michigan, 1977) has pointed out, if the communication system is to function without ambiguity then either everything or nothing must be used to transmit information in this fashion. Moreover, the organisation of the system according to symbols would also lead to a greater visual economy and an ability to make the system compact and easily manageable. A limited range of symbol/signs can be used to code a great variety of messages and would prevent the system from becoming too unwieldy if only representational images were used. Conkey (*Social Archaeology* (eds Redman *et al.*) Academic Press, 1978) has proposed that it is in the upper palaeolithic that the first pictorial symbol systems are found and which indicate that information was being organised according to this mode. The scratched motifs identified by Marshack from this period are most probably examples of such symbol systems.

But why should these hunter-gatherer societies have needed new means to exchange information about membership, status and position? Is it in fact possible to demonstrate changed social circumstances that would have selected for visual systems of communication? If we concentrate on the appearance of art and symbol systems and assume that these were a response to new social conditions then the argument becomes very circular. Where it might be possible to see changes is in the external circumstances of these societies. The first of these could relate to climatic changes at the time the art first appeared. It is first found when the climate of the last ice age turned towards much harsher, colder conditions that culminated in the

maximum extensions of the icesheets some 18,000 years ago. This would have resulted in groups becoming more dispersed as they adjusted to the new environments and the resources they contained. There would have been considerable advantages in seeking further ways of signalling membership in order to link such groups together. However this must have been a problem that faced palaeolithic groups during the earlier phases of the European ice ages and yet they did not produce art as a response. The second factor in the equation most probably concerns population. Hassan (*Advances in Archaeological Method and Theory* (ed. Schiffer) 1, Academic Press, 1978) has estimated both higher annual growth rates and greater population densities between the upper and middle palaeolithic. If this was the case then there must have been considerable pressure to hive-off population into new territories as well as to maintain growth rates at accustomed levels in just those areas that were now being

affected by climatic change. The extra information would therefore be needed to relate groups to resources and to define the extra population. Verbal communication may previously have been sufficient to exchange information but now the possibility of language differentiation would arise and this could most economically be averted by instituting a new form of communication based on visual messages that were coded stylistically. It is noticeable that at this time global population increased its range with groups colonising Australia, central Asia and hence ultimately the Americas.

The proposition that art and the intentional scratches are part of a system of communication does raise many interesting possibilities in terms of what changed in the course of human development. It also suggests that archaeologists recast their explanations for variability in the palaeolithic record toward questions concerning information rather than intelligence. □

Possible hazards of photochemotherapy for psoriasis

from Bryn Bridges and Gary Strauss

THE photosensitising psoralen drugs are being increasingly used in combination with near-ultraviolet light (PUVA) as a very effective treatment for psoriasis and other skin conditions. PUVA controls excess cell division in the skin by virtue of its ability to damage DNA. In experimental systems PUVA lesions are able to elicit all the usual consequences of DNA damage, for example inhibition of DNA synthesis, mutation, chromosome aberrations and cell transformation, as well as initiating skin tumours in mice (see Bridges *et al. Clin. exp. Dermatol.* 3, 349; 1978; Vella Briffa & Warin *J. Roy. Soc. Med.* 72, 440; 1979). The possibility that it might initiate skin cancer in humans was much in the minds of dermatologists; indeed the UK Medical Research Council has set up an *ad hoc* Working Group to consider the possible hazards of PUVA.

Although natural plant sources of psoralens have been used to treat vitiligo (lack of skin pigmentation) for thousands of years, the first report of their carcinogenicity in man followed PUVA treatment of some patients suffering from xeroderma pigmentosum, a condition in which the cells cannot repair damaged DNA effectively. Skin tumours began appearing within a month of initiation of treatment and one patient developed a lymphatic leukaemia (Reed *Acta Dermatovener. (Stockholm)* 56, 315; 1976; Reed *et al.*

Arch. Dermatol. 113, 1561; 1977). The surprisingly rapid appearance of the tumours was disturbing and unexpected for they appeared too soon after treatment to be readily explained by an increased rate of tumour initiation *de novo*. More recently an increased rate of skin cancer has been reported within the first two years of PUVA treatment of psoriasis patients (Stern *et al. N. Engl. J. Med.* 300, 809; 1979). Although the magnitude of the increase is arguable (no matched control group was used, it being a longitudinal 'before and after' comparison) it is clear that the excess tumours are associated with the previous history of the patient, either of radiation exposure or previous skin cancer.

Taking these two sets of observations together, it seems that PUVA is acting not only as a direct initiator of skin tumours (though this has yet to be established in man) but as a type of so-called 'promoter', allowing the full expression of potentially malignant foci. The idea of promoters arose from observations that some chemicals, notably the phorbol esters, although not mutagenic or carcinogenic in themselves increase the incidence of tumours if given after a very low dose of a known carcinogen (see for a short review Marx *Science*, 201, 515; 1978).

Support for the idea that PUVA, although potentially mutagenic and carcinogenic in itself, is acting in this case as a promoter comes from experiments in which ultraviolet-induced skin tumours were transplanted to syngeneic mice. The tumours grew in mice treated with PUVA but not in untreated mice (Spellman *N.*

Engl. J. Med. 301, 554; 1979).

How could such a promoter effect be operating in this case? One possible mechanism is the suppression of immunological surveillance in the skin. The idea that the body has a natural surveillance mechanism by which cells of the immune system recognise and destroy cancer cells is now generally discredited. But the skin may well be an exception to this rule. The demise of the immunosurveillance theory followed observations that there was no general increased incidence of tumours in immunosuppressed patients; however, although suppression of cell-mediated immunity by drugs such as azathioprine does not lead to a general increase in cancer, there is a rise in the number of lymphoreticular tumours and of squamous cell carcinomas of the skin (Walder *et al. Lancet*, Dec. 11, 1282; 1971; Kinlen *et al., Brit. med. J.* 2, 1461; 1979). In Stern *et al.*'s study cited above, the excess tumours were also squamous cell carcinomas whereas basal cell carcinomas predominate in the general population. This raises the possibility that PUVA may be immunosuppressive in man. In guinea pigs PUVA treatment of skin blocks delayed hypersensitivity responses in exposed areas and to a lesser but quite significant extent at unexposed sites (Morison *et al. J. invest. Dermatol.* 72, 273; 1979). The cause of the immunosuppression is as yet unknown. Roberts and Daynes (quoted by Spellman, above) have recently reported that treatment of mice with PUVA induces the development of suppressor cells. In man, a significant decrease in T cell number and function has been reported in patients undergoing PUVA therapy (Cormane *et al. J. invest. Dermatol.* 68, 253; 1977; Morison *et al. J. invest. Dermatol.* 70, 216; 1978). There is no doubt that in man circulating lymphocytes are exposed to substantial doses of PUVA when passing through the skin of treated psoriatics (see Strauss *et al. J. invest. Dermatol.* 73, 211; 1979). Furthermore, there are telling arguments in favour of the existence of resident lymphocytes in the skin (see for example, Streilein *J. invest. Dermatol.* 71, 167; 1978).

That the promoting effect of PUVA is indeed a consequence of DNA damage is indicated by rather similar immunosuppressive effects being reported for far ultraviolet light (Daynes *et al. Transplantation* 23, 343; 1978; Kripke & Fisher *Natn. Cancer Inst. Monogr.* 50, 179; 1978) and by the very much more rapid appearance of tumours observed in PUVA-treated xeroderma pigmentosum patients compared with individuals with normal DNA repair ability. The target cells for the PUVA-induced immunosuppression seen in rodents are presumably different in type from those in human psoriatic plaques that respond to the therapeutic effects of PUVA, but whether the immunosuppressive response is specific for DNA-damaging treatments or whether

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it could be elicited by other agents using other mechanisms of cytotoxicity, is an open question.

Taken together these observations suggest several possibilities of considerable fundamental as well as clinical importance. (1) Immunological surveillance may well be a reality in the skin; (2) immunological surveillance in the skin may be suppressed by DNA-damaging agents; (3) if PUVA is both a promoter and an initiator, its long-term sequelae in man may be even more severe than has hitherto been suspected.

Finally, the results with PUVA must inevitably lead to an examination of the effects of naturally occurring psoralens present in some sun-tan preparations since the activity of these in sunshine must surely result in DNA damage to the skin (Ashwood-Smith *Brit. med. J.* 3 Nov. 1140; 1979). □

The case for layered convection

from Peter J. Smith

INTEREST in the possibility of mantle-wide convection has revived recently, sparked off largely by the conclusion, derived most impressively by Cathles (*The Viscosity of the Earth's Mantle*, Princeton University Press, 1975), that contrary to previous widespread belief the viscosity of the mantle does not necessarily increase significantly with depth. With one of the most important restraints to the continuation of convection into the lower mantle removed, support for convection limited to, say, the asthenosphere is weakened, and a return to the earliest concepts of mantle-wide flow becomes possible.

Or does it? In a most thoughtful paper Richter (*J. geophys. Res.*, **84**, 6783; 1979) points out that viscosity is not the only criterion to be applied to the case for and against crust-to-core convection cells. Whatever the hypotheses about variation of viscosity in the mantle, it is an inescapable fact that earthquakes never occur below a depth of about 700 km, implying strongly that in trench zones (where the deepest earthquakes occur) the subducting lithosphere cannot pass into the region below 700 km. When an increase in viscosity with depth was accepted, this resistance to penetration seemed hardly surprising; but with that limitation removed, some other explanation of the barrier is required.

And the phenomenon is even more remarkable than a simple 700 km limit would suggest. From a new analysis of earthquakes in the Tonga-Kermadec subduction zone, Richter not only shows that all foci are shallower than 700 km but

demonstrates (using focal mechanisms) that where shocks occur below 600 km the corresponding sections of the downgoing slab become subject to downdip compression. This confirms for a particular case a generalisation made earlier by Isacks and Molnar (*Rev. Geophys. Space Phys.*, **9**, 103; 1971) and strengthens support for the view that subducting lithosphere meets insurmountable resistances when approaching 700 km depths. So, indirectly, does Richter's discovery that the seismic energy released in the Tonga-Kermadec region decreases generally with depth but then increases phenomenally between 500 and 700 km — that is, in the zone immediately above the seismic cutoff. The significance of this increase is that it more or less rules out non-barrier explanations of the 700 km limit. It is possible to argue, for example, that subducting lithosphere does pass into the region below 700 km but that by then it has become sufficiently warm to deform by creep rather than brittle fracture, thereby eliminating earthquakes. But it is difficult to imagine a deep energy peak arising from a lithosphere gradually becoming hotter with depth. Similarly, it has been suggested that lithosphere passing through the 650 + km zone changes phase, giving rise to a ductile high-density form incapable of supporting earthquake mechanisms. This proposal is more difficult to evaluate; but again the energy release peak at depth poses a severe problem.

So the 700 km limit must represent a real barrier to the passage of lithosphere, for the large energy release in the 500–700 km interval can only be explained in terms of large compressive stresses resulting from the approach towards an irresistible obstacle. What is that obstacle if it is not a viscosity increase? It could easily be chemical inhomogeneity, claims Richter, who envisages a chemically layered mantle with density increasing with depth from layer to layer. If this sounds implausible, the converse — a homogeneous mantle — sounds even more implausible, at least as Richter explains it. If the mantle is homogeneous, he argues, it must have been homogeneous right from the start or it must have become so by thermal convective mixing. But mantle conditions are such that thermal mixing will only remove inhomogeneities of less than about 1%. In other words, the conditions for the occurrence of a homogeneous mantle are more restrictive than those likely to give rise to a mantle with more than minimal inhomogeneities.

Convective systems with chemical density variations have been studied in considerable detail in seawater. Extending the principles thereby derived to the mantle, Richter concludes that a significantly inhomogeneous mantle will develop layering in which convection is restricted to the discrete layers. Precisely how many convective layers there are in the mantle will depend on how the Earth

accreted and how the mantle developed originally as the residue left by the removal of crustal and core materials — and these factors are uncertain. But since the mantle is more likely to be inhomogeneous than homogeneous, it is more likely to have more than one convective layer than it is to have just one. So, although whole-mantle convection is a plausible option again thanks to revised views about the mantle's viscosity, the details of the convection could be rather different from those envisaged by the early supporters of the idea. □

Antigenic shift and drift

from a Correspondent

INFLUENZA has been relatively dormant as a human disease for the past several years. However with the advent of recombinant DNA technology and of monoclonal antibodies, work on the influenza virus has burgeoned. Particularly exciting are the recent advances in knowledge of the structure of the viral haemagglutinin (HA) and of the mechanisms which allow its antigenic variation and the consequent emergence of new viral strains. So it was not surprising to find that this molecule was of central interest at a workshop* held early in December near Canberra, Australia.

HA, the major glycoprotein of the viral envelope (molecular weight 75,000) is coded by an mRNA specified by one of the eight segments of RNA present in the virus particle. Pandemics of influenza occur in the human population when a major shift in the antigenic structure of HA allows the virus to infect people who are immune to previously circulating strains. Obviously one would like to compare the complete primary amino acid sequence of HAs of several different pandemic strains. However, despite considerable effort by the protein chemists (M. Waterfield, ICRF; C. Ward and T. Doppeide, CSIRO, Melbourne) such information has proved difficult and laborious to obtain. Consequently the nature of the variation involved in the creation of new pandemic strains has remained unknown. As reported at the workshop, several groups now have cloned in prokaryotic vectors, full length cDNA copies of the HA genes from viruses belonging to two of the three major pandemic groups. Complete DNA sequences have been established for the Asian H₂ strain, which first emerged in 1957 (M.-J. Gething, ICRF); for several members of the Hong Kong series which first appeared in 1967 (N. Sleight & G. Both, CSIRO, Sydney; W. Min Jou, Ghent; G. Threlfall, Searle) and for the Rostock

*An International Workshop on 'Structure and Variation in Influenza Virus', organised by Graeme Laver and Gillian Air, Australian National University, Canberra, Australia.

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strain of avian influenza virus (A. Porter & J.S. Emtage, Searle, High Wycombe). In all cases the general organisation of the HA gene is remarkably similar. There are untranslated regions (albeit of varying lengths) at both 5' and 3' ends of the gene. The 13 nucleotides which lie at the 3' ends are identical in all viruses so far examined and are presumably used in replication (J. Skehel, NIMR, London); the remainder of the untranslated regions show little evidence of common origin or function (G. Air, Australian National University, Canberra). In all cases the coding portion of the gene exists as a continuous block of nucleotides so that removal of intervening sequences apparently is not involved in the expression of HA. However the mRNAs of influenza are known to sequester 5'-capped termini from nascent or newly-synthesised cellular RNAs, and splicing, or something like it, may play a part in this transfer (R. Krug, Sloan-Kettering Institute, New York).

The sequence of amino acids, deduced from that of the DNA, confirms that HA invariably contains three distinct hydrophobic regions. One of these forms the N-terminus of the primary translation product. It is cleaved from the mature protein and is believed to be responsible for insertion of the nascent polypeptide into the membrane of the endoplasmic reticulum. A second hydrophobic region located at the C-terminus is involved in anchoring the mature HA in the viral envelope. Both of these hydrophobic regions show a surprising degree of variation and it seems that hydrophobicity *per se* is more important than primary amino acid sequence for interaction of HA with membranes. By contrast, the amino acid sequence of the third hydrophobic region which lies roughly in the centre of the molecule is absolutely conserved between viral strains. Ultimately it forms the new N terminus of the smaller subunit of HA (HA2) which is created by proteolytic cleavage of the full length polypeptide. This cleavage, which is necessary for infectivity of the virus, is thought to allow the third hydrophobic region to interact with the membrane of the target cell. Approximately 60% of the amino acid sequences of HA2 are conserved between strains. The remainder of the molecule (HA1) shows much more extensive variation. Nevertheless, because the locations of bridging cysteine residues in both HA1 and HA2 are totally conserved, it is likely that the three-dimensional structure of HA (currently under investigation, D. Wiley, Harvard University) will turn out to be very similar in different viral strains. These results are consistent with the idea (D. Jackson, University of Melbourne) that the major antigenic sites are located in HA1 and that it is variation in these sites which allows the virus to escape from neutralising antibody. However the results reported at the workshop show the HA1s of different

pandemic strains to have diverged to such an extent that it is impossible to map their sites of antigenic difference merely by comparing their amino acid sequences. Furthermore the fact that the HAs of two human strains are no more similar to each other than to the HA of the avian strain means that it is impossible to establish a genealogical tree which would define the evolutionary relationship between different pandemic strains.

At present therefore, it is impossible to decide between the two alternative hypotheses which attempt to explain how new pandemic strains of influenza virus arise. One suggestion, for which circumstantial evidence exists (see Laver & Webster *Brit. Med. Bull.* 35, 29; 1979) is that new pandemic strains are recombinants formed between influenza viruses of humans and those of domestic or wild animals. The second possibility is that the emergence of 'new' pandemic strains in the human population represents no more than the reappearance in essentially unchanged form, of viruses that had circulated years previously, and then had entered a latent state in humans or lower animals. Although such a latent state is entirely conjectural, this idea has received serious consideration because of the reappearance in 1977 in China of a strain of influenza virus that was in wide circulation until 1950 and then had disappeared. The re-emergence of this strain adds an entirely unexpected complication to both the epidemiology of influenza and the evolution of the virus. It implies that influenza virus, at least in some circumstances, in some hosts, can preserve its antigenic structure for long periods of time. Such is not the general case in humans.

Between the major shifts in antigenic structure which define the beginning and end of each pandemic era, the virus undergoes a series of smaller changes. As the human population becomes immune to infection by extant strains of influenza virus, so the pressure rises to select variants which by displaying small but significant changes in antigenicity can evade the immune response. This process is known as antigenic drift.

The changes which are responsible for antigenic drift accumulate with time, and field strains isolated several years apart from within a single pandemic era show a surprising number of nucleotide and amino acid substitutions (Both), in one case 20 amino acids in 9 years (Air & Laver). Presumably some of these substitutions affect viral antigenicity. However it is also possible that some of these changes may be antigenically neutral or may cause antigenically significant conformational alterations in distant parts of the HA molecule. Therefore, it has not been possible so far to correlate the locations of antigenic sites with the positions of amino acid substitutions.

To simplify the system, several groups

have attempted to mimic antigenic drift *in vitro*. The virus is grown in the presence of large quantities of either high titre neutralising antibody (B. Moss, CSIRO, Sydney) or more recently, monoclonal antibodies directed against single antigenic sites in HA (R. Webster, St. Jude's Children's Hospital, Memphis; Laver; W. Gerhard, Wistar Institute, Philadelphia). Variants which are resistant to inactivation by the antibodies arise at a frequency of 10^{-5} to 10^{-6} . Peptide mapping and nucleotide sequencing show that such variants arise as a consequence of changes limited in both number and location within HA1. Thus in 10 variants selected with monoclonal antibodies, a proline at position 143 of HA1 has changed to serine, threonine, leucine or histidine. The simplest interpretation of this result is that the region around position 143 is at or near an antigenic site. This is consistent with the finding that field strains which presumably have been undergoing a similar selection process *in vivo* often display changes in the same region of the protein, although for reasons which are not clear the proline at position 143 is invariant.

As stated above, the field strains show alterations in many additional regions of the HA molecule. Thus the total number of antigenic sites may be greater, their topological relationship more complex and the selection pressures which mould them more subtle than we at present appreciate. Nevertheless the capacity to select a sequential series of isogenic mutants and analyse them rapidly at the molecular level is an important advance that represents the best hope of dissecting the process of antigenic drift. □

Man in space

from Anthony Goode

MANNED space flight now spans almost two decades but it began amidst considerable scepticism; an Astronomer Royal summed up a widely held belief by stating, somewhat incautiously, that space travel was utter bilge, and certainly in 1961 extensive and catastrophic effects of exposure to weightlessness were predicted. Despite this, man is now firmly committed to space. Exploration of its full potential depends on his well-being in this unique environment. The reusable Space Shuttle, whose first flights are planned for 1980, will open up space to a wider range of users, including the working scientist and provide a chance to assess more clearly the potential medical problems.

Cardiovascular changes

Although the catastrophic effects of exposure to weightlessness have not materialised gross changes do occur in the

bodies of astronauts. Going up into orbit immediately induces a massive redistribution of about 2 litres of blood and interstitial fluid into the head and thorax causing venous distension. The face becomes puffy whereas the legs shrink. This redistribution directly influences cardiac activity and indirectly, the control of the circulation, and produces changes in the clearance of salt and water through the kidneys. This thoracic engorgement persists in flights of up to 84 days giving rise to fears about the longer term possible consequences of cardiac hypertrophy and arterial hypertension. The changes however, do not affect work capacity in orbit. Return to Earth results in a decline in circulatory tolerance to an erect posture, reduced cardiac output and stroke volume and a decreased capacity for physical work. After Skylab flights these changes were reversed to normal pre-flight values within 10 days. The precise degree of thoracic engorgement and how close the pressure levels in the pulmonary artery come to inducing pulmonary oedema will be investigated early in the Space Shuttle series.

Changes in red cell morphology have been found after spaceflight together with loss of up to 8–10% of red cell mass after Apollo and Skylab missions. The reason for this is obscure; chromium-51 labelling of red cells has shown no increased destruction. Cell proliferation in tissue culture is normal in flight and the cellular and hormonal components of the immune response *in vivo* remain unchanged. Renal function is satisfactorily maintained but a possible diuresis early in flight is accompanied by a fall in antidiuretic hormone and an increase in aldosterone with paradoxical solute excretion.

Bone and muscle

In the first days in orbit there is a 3–4 kg loss in body weight as a result of loss of body fluid and, to a lesser extent, a reduced fluid intake. After a longer period in flight, 2–3 kg are lost, mostly as body muscle, although this loss may be reduced by about 50% by an intensive exercise regimen. The loss does not therefore simply reflect muscle atrophy, but no additional cause has yet been found. Bone calcium is lost continuously at a rate of 0.5% of total body calcium per month, about three times the loss induced by prolonged bed rest. Apart from increasing the risk of renal calculi in astronauts, this excretion could limit flights to about a year's duration before bones become brittle to support the body on Earth. Rigorous exercise regimes, hormone analysis and dietary measures have failed to elucidate the underlying cause, and muscle and bone metabolism will be an area of intensive future investigation.

Man has a well-developed skeleton and musculature suited to Earth's gravity but such attributes may be an embarrassment in space. One observer has speculated that

young amputees may be ideal crew members for longer flights, as they would be subject to less fluid shift and mineral depletion.

Sensory systems

On Earth man maintains his sense of balance by the gravity-sensitive vestibular system in the ear, which together with the somatosensory and visual systems allow spatial orientation and the control of posture and locomotion. However, in zero *G* the gravity-sensitive otoliths of the vestibular system fail to indicate the orientation of the head as indicated by the other sensory mechanisms and this mismatch causes a perceptual conflict during movement, either of the whole body or of the head alone. In zero *G* astronauts have experienced perceptual deficiencies or illusions such as a compelling sensation of flying upside-down or of turning or tilting when they move their head rapidly. Skylab astronauts reported that they were less able than normal to locate objects relative to their body when their eyes were closed.

Early flights in Mercury and Gemini capsules in which astronauts were restrained on couches and unable to move their body and head freely prevented the space motion sickness which had been reported during the Russian Vostok flights. However, during Apollo and Skylab missions half the crewmen developed sweating, pallor, nausea and vomiting, which began within minutes to hours of their starting to move around in the capsule and which remitted within 3 to 6 days. The phenomenon is probably due to intersensory mismatch and once the body has become accustomed to the new environment, head movements no longer cause nausea. Anti-motion sickness therapy may relieve the symptoms but the undesirable sedative side effects can result in less efficient performance during critical early phases of the mission. NASA has selected an experiment for Spacelab 1 in which astronauts will be exposed to a selection of motion sickness tests first on Earth and then in zero *G*. These tests should provide useful predictions of individual susceptibility to space motion sickness. The European Space Agency (ESA) has designed a linear accelerating stimulus device — the 'space sled' — which will allow studies of human sensory physiology, measurement of perceptual thresholds and analysis of eye movements and muscle activities affected by defined rectilinear accelerations.

Radiation hazards

Certain components of cosmic radiation may be a hazard to man in space. The Apollo 11 crew saw faint spots and flashes of light every half minute or so on the return flight from the Moon. This was the result of HZE particles (of high charge and

energy up to 10^{14} MeV) penetrating the spacecraft and the astronauts' eyes and producing visual sensations after interaction with the retina. The 'Biostack' experiments were designed with monolayers of biological objects sandwiched between nuclear track detectors, and demonstrated serious biological effects. The most arresting results with *Artemia salina* eggs showed that the hatching rate was reduced, that HZE particle collision with nauplius larvae caused abnormal growth and behaviour with a high mortality after hatching, stunted growth, delayed sexual maturity and reduced fertility. Advanced 'Biostack' experiments are designed for Spacelab to investigate this phenomenon which may pose a danger to non-regenerating human tissues particularly those of the central nervous system.

Space Shuttle

The reusable Space Shuttle will allow life scientists to work on orbit in a shirt sleeve environment for up to 30 days. The payload specialist will have a gentler ride than in the Saturn/Apollo system as now the maximum gravity load will be 3*G* and he will be content in the knowledge that a rescue capability is available. With less physical stress during flight and no direct flight responsibilities it is hoped that medical criteria for payload specialists will be much less strict than those applied to astronauts although this has not been the case with the initial selection.

A legacy from Project Apollo has been a feeling, reflected in government thinking, that spaceflight is remote from reality and too expensive. A policy of selecting life sciences payload specialists principally for their professional abilities may help to overcome this, improve the quality of research and in turn contribute to the safe, effective and extensive use of space. Both NASA and ESA are properly dependent on budget allocations from the member state governments and recent monetary restrictions may reduce the frequency of flights dedicated to the life sciences. This parsimonious attitude is perhaps surprising as astronomy, from the days of Stonehenge an interesting but costly diversion with little direct impact, is well funded. It is perhaps salutary to remind government agencies that space flight provides an important stimulus for diverse investigations with unpredictable results.

Shuttle flights will probably begin in 1980. The initial submission of life science experiments has been good and indicates considerable interest in the life science community. Finance for experiments may be more difficult but this system offers an opportunity to study the behaviour of biological systems in a new environment, and the applications are potentially unlimited. We should all now consider more carefully how we can use the unique properties of space to complement and enhance current research projects. □

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REVIEW ARTICLE

Three-receptor, clonal expansion model for selection of self-recognition in the thymus

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The postulate is made that dual recognition by T lymphocytes is due to two types of receptor, one encoded by antibody genes and one by a distinct multigene family with simple rules for expression. This postulate leads to a model explaining ontogenic and evolutionary selection for self recognition, T lymphocyte effector function, the apparent high frequency of alloreactive T cells and immune response gene activity. The model is contrasted with previous explanations of self-recognition phenomena.

ALL of the cell surface products coded for by genes of the major histocompatibility complex (MHC) have a crucial role in the regulation of the immune response. The cell surface products of the mouse H-2 complex have been divided into two classes: (1) those originally recognised as transplantation antigens (K and D); and (2) those recognised as immune associated (Ia) antigens (encoded at the H-2 I region). Initially I-region genes were shown to be important in regulating immune responses but more recently K- and D-region gene products have been shown to be involved in recognition and killing by T lymphocytes of cells that display foreign antigens on their surface. Thus cytotoxic T cells which have been generated in response to virus-infected cells¹, hapten-modified cells² or minor histocompatibility antigens³ are only capable of lysing those target cells which bear the same foreign antigen and the same major histocompatibility antigens as the immunising cells. This phenomenon has been termed major histocompatibility restriction (thus for the mouse H-2 restriction) of cytotoxic T-cell responses.

Immune responses other than those involving cytotoxic T cells also show H-2 restriction. The proliferative response of T cells to antigen presented on the macrophage surface requires histocompatibility at the H-2 I region of the two cell types⁴. Similarly cooperation between helper T cells and B cells exhibits H-2 I restriction⁵; the fact that in chimaeric animals H-2 incompatible T and B cells are able to cooperate^{6,7} being explained by the hypothesis that lymphocytes can adaptively differentiate, that is, develop the ability to recognise cell interaction molecules on allogeneic cells⁸. To explain the various types of H-2 restriction two types of hypothesis have been proposed⁹.

The dual recognition hypothesis states that T cells exhibit two separate receptors on their surfaces, one of which shows specificity for H-2 antigens, the other for non H-2 antigens. In one form of this hypothesis the T-cell receptor for H-2 is itself an H-2 coded product whereas the receptor for foreign antigens is presumed to use the antibody gene repertoire¹⁰. In an alternative form of the hypothesis the two receptors are identical at an early stage in development and must therefore be encoded either by the H-2 linked gene system or by the antibody gene repertoire¹¹.

The altered self hypothesis postulates that T cells bear a single receptor which is only capable of recognising antigens in association with H-2, for example, new antigenic determinants created by the interaction of the foreign antigen with the H-2 antigen. In most models immunoglobulin variable region genes (V genes) have been assumed to code for the receptors.

Some form of dual recognition hypothesis is most attractive. The evidence for I-region restricted systems is more easily compatible with a dual recognition hypothesis than with an altered self hypothesis. Also, for the cytotoxic T cell, the dual recognition hypothesis has recently emerged as the favoured candidate. Indeed it has been argued forcibly that there is no viable form of altered self hypothesis¹⁸.

In the initial demonstration of H-2 restriction the evidence was consistent with the idea that the immunising or target cells presenting the foreign antigen, needed to share histocompatibility antigens with the cytotoxic T cell. Thus restriction was seen as synonymous with a recognition of self antigens. Subsequently it was demonstrated that identity of histocompatibility antigens between immunising or target cell and the cytotoxic T cell is not necessary for recognition of self. The T cell 'learns' to recognise as self the histocompatibility antigens displayed on the epithelial cells of the thymus during the processing of T-cell precursors through that organ. This finding stems from experiments involving the use of bone marrow chimaeras and grafts of irradiated thymus tissue. Bevan¹² used chimaeric mice constructed by injecting (A × B) F₁ bone marrow into an irradiated parent A recipient to show that cytotoxic T cells generated against minor histocompatibility antigens recognise these antigens preferentially in association with the H-2 haplotype of the parent A, as opposed to that of parent B. Zinkernagel *et al.*¹³ constructed similar chimaeras and showed exclusive restriction for the H-2 type of parent A in cytotoxicity of either virally infected or hapten-modified target cells. Chimaeric mice constructed by injecting (A × B) F₁ bone marrow into irradiated (A × C) F₁ recipients were constructed and the lymphocytes sensitised against (B × C) F₁ cells chemically coupled to the hapten trinitrophenyl (TNP). In this case cytotoxicity was restricted to TNP-modified cells of the C haplotype. Thus T cells heterozygous for the haplotypes of A and B parents have learnt to recognise the H-2 type of the C mouse as self but failed to recognise the H-2 type of the B mouse (genotypically a self haplotype) as self. The use of irradiated thymus grafts in adult thymectomised irradiated recipients of bone marrow cells showed that the H-2 type of the thymus epithelium determines what the newly generated T cell learns to recognise as the self H-2 type¹⁴.

Self recognition is clearly not determined by the identity of the H-2 haplotype between T cells and target cells. Zinkernagel *et al.*¹³ pointed out that the learning of self recognition in the thymus is best explained on the basis of a dual recognition hypothesis because the altered self hypothesis would require the special rule that the specificities generated by modified self in thymus A do not overlap with the specificities generated in thymus B. However, the authors made no clear prediction as to whether dual recognition is achieved by two separate receptor molecules or two parts of a single receptor molecule.

These experiments and hypotheses had been influenced greatly by Jerne's prediction¹⁵ of a role for the thymus in the selection of T-cell specificities. The model proposed by Jerne took as its starting point an inherited set of T-cell receptor antibodies each specific for an allelic form of one of the major transplantation antigens of the species, with the total repertoire reflecting the extent of polymorphism of the histocompatibility antigens of the species. The model then invoked rapid somatic

mutation of the genes coding for receptor antibodies. Cells carrying receptors for non-self alloantigens were not eliminated in the thymus and emerged into the periphery in the high proportions expected for the products of germ line structural genes. These clones of cells explained the high frequency of allo-reactive T cells. T cells maturing in the thymus and expressing anti-self receptors were assumed to be eliminated unless those receptors mutated. In this way rapid somatic mutation was postulated to generate the total repertoire of receptors for all foreign antigens from the inherited genes for anti-self. This hypothesis was subjected by Bodmer¹⁶ to the major criticism that individual members of a species could not be aware of the extent of polymorphism of the histocompatibility genes within the species. How then could there be a sufficient selective pressure for each animal to carry germ line genes coding for antibodies against the total repertoire of histocompatibility alleles? A second major criticism of the Jerne model for the generation of antibody diversity lay in the apparent difference in receptor repertoires between T and B lymphocytes. In particular it was not possible to detect the predicted high proportion of allo-reactivity at the B-cell level.

In spite of these major objections to the Jerne model it did predict the crucial selective role for the thymus that has been demonstrated recently. This has led Jerne and his colleagues¹¹ to propose a modification of the somatic mutation hypothesis for clonal selection within the thymus. Their modification involved two identical anti-self receptors, allowing mutation and selection to act on only one of them. This modification requires that special genetic mechanisms be invoked in addition to rapid somatic mutation. Other hypotheses explaining T-cell specificity in terms of V gene products also require rapid somatic mutation or rules of V gene expression unique to T cells¹⁷⁻¹⁹. I have therefore devised an alternative model for the selective function of the thymus and the reactivity of T lymphocytes.

Three-receptor, clonal expansion model

It is the main purpose of this article to propose a clonal selection hypothesis for the generation of T cells in the thymus that emphasises clonal expansion (rather than clonal deletion as in the Jerne model) and does not require somatic mutation.

The starting point for this model is the need to explain the basis for 'learning' of self from the phenotype of the thymus rather than from the genotype of the pre-T cell. It is proposed that the genotype of the pre-T cell must contain the information for a receptor molecule that can recognise allo-H-2 antigens as self and that this receptor is not an antibody molecule.

The model takes dual recognition as the basis for H-2 restriction and T-cell specificity and states that dual recognition is effected by two distinct receptor repertoires encoded by separate sets of genes. One type of T-cell receptor is encoded by immunoglobulin variable region genes and embraces the same repertoire and similar rules of expression as B-cell antibodies. The postulated second set of T-cell receptors is distinct from immunoglobulins, but has a repertoire of specificities similar to that originally described for immunoglobulin variable region V genes in the Jerne model¹⁵ (but with contrasting consequences).

If pre-T cells of each genotype can recognise any thymus phenotype as self through an appropriate non-immunoglobulin receptor, then it follows that each animal carries genes coding for a repertoire of receptor molecules capable of recognising the repertoire of allo-antigenic specificities of major histocompatibility antigens. Thus receptors $R_1R_2R_3 \dots R_n$ will recognise respectively the MHC antigens $H_1H_2H_3 \dots H_n$.

The genes coding for these histocompatibility antigen receptors (R anti-H) are assumed to constitute a multigene family present in all members of the species. It is postulated that expression of genes in this family is limited to a single gene per haplotype in each cell, with no requirement for allelic exclusion. Expression of R anti-H occurs initially on pre-T cells. Each pre-T cell will express two R anti-H molecules selected at random from the inherited repertoire. A simple matrix of cell

specificities is shown in Fig. 1. The combinations of R anti-H molecules on pre-T cells will be, in order of frequency: (1) both specific for allo-MHC antigens (R anti-allo-H); (2) one specific for a self-MHC antigen (R anti-self-H); (3) both specific for self MHC antigens. Examples of these three cell types in the matrix (Fig. 1) are: (1) R_2R_2 ; (2) R_1R_2 ; (3) R_1R_1 .

Within the thymus the epithelium provides MHC antigens to interact with the R anti-H molecules on pre-T cells. The presence of at least one (or occasionally two) R anti-self-H molecules on a pre-T cell allows interaction with self-MHC antigens and this is postulated to give a proliferative stimulus to that pre-T cell (Fig. 2). Clonal expansion generates mature T cells all of which carry a receptor for a self-histocompatibility antigen. Because of the random diploid expression of R anti-H molecules the expanded population of T cells will be subdivided, in approximately n equal proportion, into n clones carrying either a second R anti-self-H or a single R anti-allo-H molecule. If we make the simple assumption that there are 100 R anti-H genes corresponding to 100 distinct allo-H specificities, then 1% of all T cells will carry one R anti-allo-H molecule corresponding to any particular allo-H specificity. The clonally expanded population of T cells will be exported from the thymus to the periphery. Pre-T cells which do not express at least one R anti-self-H molecule will not receive a proliferative signal leading to clonal expansion; such cells, if not eliminated, will be diluted out.

During the clonal expansion of pre-T cells driven by the interaction of R anti-self-H with self-H antigens each cell becomes committed to the expression of a particular receptor antibody molecule using the same repertoire of V genes encoding B-cell antibody molecules (Fig. 2). In B-cell development commitment of the pre-B cell to a single antibody specificity demands allelic exclusion and κ/λ exclusion. This exclusive commitment is probably effected by rearrangement of gene segments involving only two of the six V gene families present in the diploid cell¹⁸⁻²⁰. For simplicity in the present model it is assumed that the differentiating T cell makes the same commitment as the differentiating B cell. Thus each T cell will express at random a single antibody V gene coded receptor molecule (whether both V_H and V_L contribute to this receptor is not important for the model). The entire repertoire of V gene encoded receptors will be expressed on individual T cells within each of the n expanding clones of pre-T cells defined by expression of pairs of R anti-H receptor molecules. It is necessary to invoke as a developmental limitation that when T cells become committed and express a V gene encoded receptor they cease being sensitive to the proliferative signal driving clonal expansion via the R anti-self-H receptor.

The postulated developmental limitation is simply explained by postulating an interaction between R anti-H and V anti-X. This interaction, or coupling, ensures that two signals are now required to stimulate the mature T cell. Because it is necessary to introduce a self tolerance selection acting on V anti-X this could be accommodated by the proposal that if V anti-X encounters X before the coupling of R anti-H and V anti-X the cell will be eliminated and tolerance will result. This tolerance model could involve dual recognition of X and self-H as proposed previously (ref. 23 and J. M. Phillips-Quagliata, personal communication).

Each mature T cell entering the periphery will carry three receptor molecules: (1) R anti-self-H; (2) R anti-allo-H (100/ n % of the cells will carry second R anti-self-H); (3) a V gene encoded receptor antibody (Fig. 2). Restriction of responsiveness of T cells in the periphery will be accounted for if it is required that dual recognition involving R anti-self-H and a receptor antibody specific for the foreign antigen (X) is necessary for triggering an immune response. Restriction will be limited effectively to self-H specificities as determined by the R anti-self-H present on all T cells and selected in the thymus, but independent of the self-H type present on peripheral cells. A small subset of T cells will carry R anti-allo-H receptors capable of being involved in dual recognition of antigens presented together with allo-H specificities but, in the numerical example

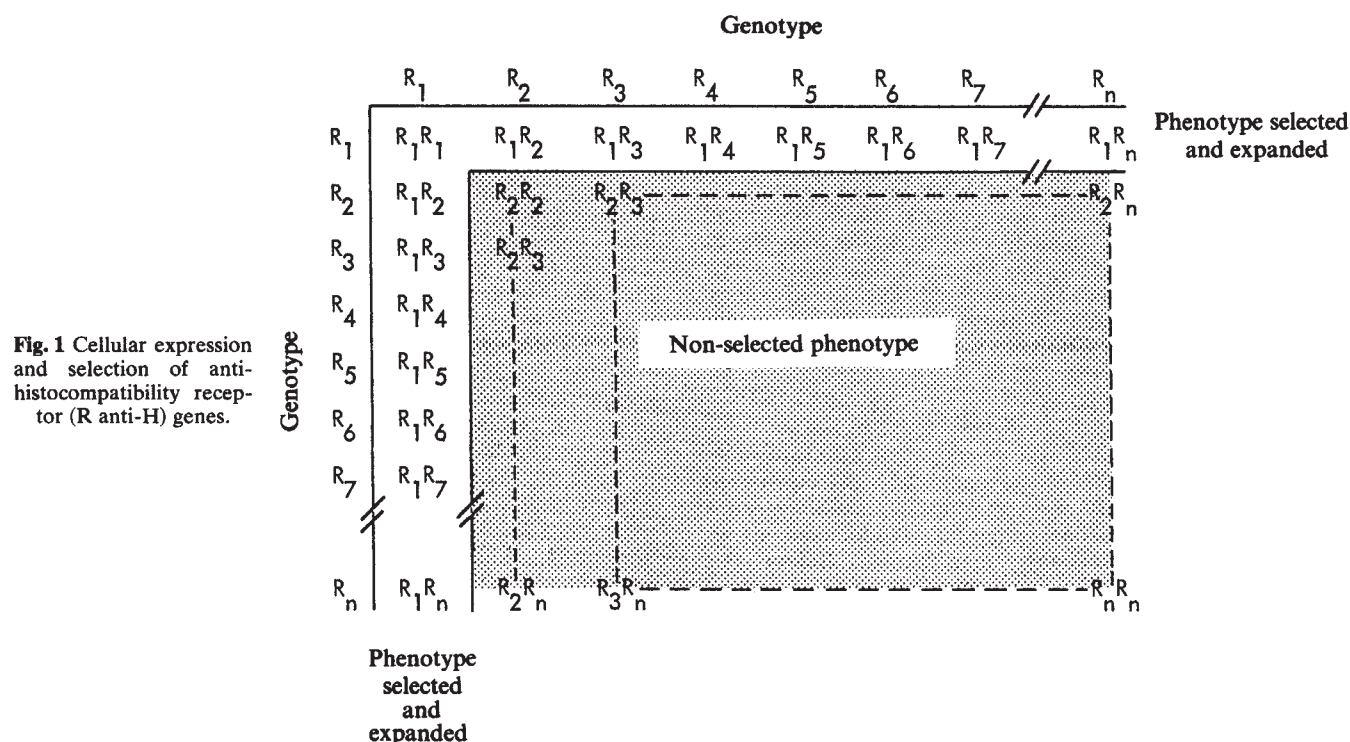


Fig. 1 Cellular expression and selection of anti-histocompatibility receptor (R anti-H) genes.

quoted above, such antigen presentation with allo-H specificities could only evoke a response at the 1% level relative to self-H presentation. However, in a situation where self presentation is not possible clonal expansion of this minor population of T cells could explain responsiveness²⁴.

Evolution of the R anti-H multigene family

One of the basic tenets of the present hypothesis is that each member of the species has the genetic capacity to make R anti-H molecules specific for the species repertoire of allo-H antigens. This parallels the specificity repertoire envisaged for germ line V genes in Jerne's hypothesis¹⁵. In that case, Bodmer¹⁶ argued that it was almost impossible to conceive of a selective mechanism which could be powerful enough to maintain a situation requiring parallel evolution of histocompatibility antigens and immunoglobulin genes. Why then in the present model should a multigene family coding for R anti-species-H specificities be considered to be a feasible starting point. To answer this question we must look at the evidence which leads one to invoke such a multigene family and the selective pressures which could have led to the evolution of the gene family.

The unexpected ability of pre-T cells to 'learn' to recognise as self the phenotype of the thymus epithelium, even when this phenotype is at variance with the genotype of the pre-T cells, leads to the logical postulate that the genotype of one strain codes for receptor molecules able to recognise histocompatibility antigens of another strain. The limitations to this allo-recognition process can only be uncovered by further experimentation. The evidence also indicates that it is essential for immune responsiveness that each animal exhibits complementary R anti-H and H molecules¹⁴. If the genes coding for R anti-H and H are segregating independently and the H antigens are highly polymorphic there will be a selective pressure for the accumulation in the R anti-H gene family of genes coding for all possible R anti-H specificities. Genes coding for a complete (or extensive) repertoire of R anti-H specificities will be maintained in the germ line of the species because of the strong selective pressure for each member of the species to be able to recognise its own histocompatibility antigens as self. It is important to this argument that gaps in the immune responsiveness of T cells due to an absence of certain R anti-self-H genes in a given member of the species will lead to an increased susceptibility to viral infection and therefore an increased probability of the elim-

ination of that individual before reproduction. There will be a strong selective pressure, enhanced by any polymorphism of the R anti-H genes, for MHC heterozygosity to maximise the chance that self can be recognised.

The multigene family coding for R anti-species-H specificities is postulated to be distinct from the immunoglobulin gene families and is not postulated to be encoded at the MHC as previously invoked for the T-cell receptor. However, it is important to realise that the expression of particular R anti-self-H genes will be controlled by the MHC so that the R anti-H T cell receptor will appear to be encoded at the MHC.

It might be expected, on the basis of the known specificity of restriction, that each R anti-H recognises a private histocompatibility antigen. This prediction explains the finding²⁵ that a private (and not a public) H-2 specificity is recognised in the restriction of a cytotoxic response to virus infected cells although public H-2 specificities are recognised in anti-allo-H-2 responses, both cytotoxic and proliferative. In both responses the private H-2 antigen is postulated by the present model to interact with R anti-H. In the anti-viral response the V receptors will recognise viral antigens while in the anti-allo-H-2 response the V receptors will be specific for both private and public H-2 antigens. It should be possible to define the repertoire of R anti-H specificities from the restriction of responses by private H-2 specificities.

T-cell effector types

The hypothesis automatically accounts for the finding that separate populations of T cells recognise antigen in association with each histocompatibility antigen (for example, in the mouse T cells are specific for either K, D or I antigens). The model is consistent with the division of T cells into subsets having different effector functions but each displaying a full repertoire of specificities^{26,27}. The expression of R anti-K or R anti-D will lead to T cells destined to become cytotoxic cells, while the expression of R anti-I will lead to T cells destined to become helper cells. The model predicts that individual clones of cytotoxic T cells will show either K or D restriction²⁸⁻³⁰. This specificity will be provided without the need to invoke separately controlled repertoires of genes coding for R anti-K, R anti-D and R anti-I. A single multigene family containing an assortment of genes coding for each of these receptor types will, on the basis of the random expression of one receptor per

haploid set per cell, infrequently give rise to a cell expressing two receptors with specificities for antigens of the same haplotypes. Most cells will display one R anti-self-H together with one R anti-non-self-H. Allelic exclusion of R anti-H expression is not necessary for clonal restriction to K, D or I recognition.

The small percentage of T cells displaying two different R anti-self-H specificities would not be expected to make a significant contribution to immune responsiveness, which will therefore be effectively clonally restricted for a single H specificity (K, D or I). However, the small fraction of T-cell clones with any given third receptor for R anti-allo-H will be able to recognise the foreign antigen in association with that allo-H specificity. Such a subset of T-cell clones has been found in limiting dilution experiments³¹.

Frequency of allo-H-2 reactive cells

One of the major problems which any model for T-cell receptors and self recognition must explain is that of the high frequency of cells showing specificity for allo-histocompatibility antigens³²⁻³⁴. The present clonal expansion model contains an inherent explanation for this phenomenon. Before this explanation is discussed, the nature of the problem should be clarified. There may well be both a qualitative and a quantitative difference in the perception by T cells of H-2 antigens relative to all other antigens. The absolute frequency of allo-H-2 reactive cells is clearly high but is a difficult figure to establish with any accuracy. Frequently allo-H-2 reactive cell frequencies in excess of 1%³⁵⁻³⁸ are compared with frequencies of 10^{-6} – 10^{-5} for cells responsive to conventional antigens (the latter figure appears to be drawn from data on the specificities of B cells). When precursor cell frequencies for allo-H-2 reactive cytotoxic T cells determined in a limiting dilution assay were compared with the frequency of cytotoxic cells specific for a hapten (TNP) the frequency of hapten responsive cells was of the order of tenfold (between eight- and thirty-fold) less than the frequency of alloreactive cells³⁷. Moreover when the allo-H-2 difference was restricted to either the K or D end of H-2, the frequencies of reactive cells were comparable with those specific for TNP. It is therefore possible that the major difference in frequency of antigen specific cells is between the B-cell and T-cell populations rather than between the frequency of allo-H-2 reactive T cells and T cells of all other specificities. In this regard it is noticeable that B-cell responses to H-2 allo-antigens are much less pronounced than corresponding T-cell responses.

With the above discussion in mind, let us look at the receptor specificities predicted by the present model to be present on cells reactive with allo-H-2 antigens or other foreign antigens (designated X). T cells reactive to X must display R anti-self-H (K, D or I) together with a V receptor specific for X. The V receptor repertoire on B cells and T cells is identical but it is reasonable to expect that with dual recognition there will be activation of T cells possessing V receptors with lower affinity for X than is necessary for activation of a B cell. This difference in affinity threshold could account in part for the apparently higher frequency of T cells reactive with X relative to B cells reactive with X. T cells reactive with allo-H-2 differences will need to possess R anti-allo-H (K, D or I) together with V receptors specific for H-2 allo-antigen differences. In the case of responses limited to differences at K or D loci the frequency of reactive cells would not be expected to be higher than the frequency of cells reactive to X. However, many measurements of allo-reactivity have been made using strains of animals different at multiple loci (major and minor histocompatibility differences). In such cases a much bigger proportion of T cells bearing R-anti-allo-H molecules could be activated because the repertoire of V receptors specific for all other antigenic differences would be susceptible to stimulation in a dual recognition manner.

Passive selection systems involving the absorption of T cells by exposure to monolayers of histoincompatible cells³⁹ or by passage through histoincompatible animals⁴⁰ have been used to assess the fraction of T cells binding a particular antigen. In

posing the problem of the proportion of allo-H-2 reactive cells the frequencies determined in these passive selection assays should be clearly distinguished from the frequencies determined in assays for precursors of effector cells. It should be remembered that in the B-cell system the frequency of antigen-binding cells is often found to be 10- to 100-fold greater than the frequency of B cells acting as precursors for specific antibody-producing cells⁴¹. It is reasonable to assume that V receptors behave similarly on both B and T cells and that a difference in frequency between antigen-binding cells and antigen reactive cells might be seen for T cells as it has been for B cells. In the present model this would imply that a considerable proportion of T cells carrying R anti-allo-H-2 might be removed in passive selection. In addition cross reactivities at the level of R anti-H receptors, not detectable at the level of responding cells, might be revealed by passive selection methods.

The percentage of cells responding in allo-aggression has led to the suggestion, and to evidence supporting that suggestion^{32,42}, that T cells are not monospecific as required for clonal selection. In the present model the T cells responding in allo-aggression are monospecific (monoclonal) with respect to R anti-allo-H specificity but that population comprises a multiplicity of T cells each monospecific with regard to its V region coded receptor and each expanding in an antigen-driven monoclonal fashion.

In addition to containing an inherent explanation of allo-reactivity the present model appears to offer explanations for a number of other phenomena.

T-cell receptor idiotypes and antibodies to T-cell receptors

Considerable evidence is accumulating to show that T cells specific for either foreign antigens or alloantigens have receptor molecules carrying idiotypes identical to B-cell antibodies specific for the same antigens^{43,44}. It is axiomatic to the present hypothesis that these idiotypes are encoded by the same genes.

The presence of identical idiotypes on B and T cells allows for the physiological action of anti-idiotypic antibody on both sorts of cells postulated in an idiotypic network hypothesis⁴⁵. It is reasonable to suppose that the R anti-H family of molecules, unlike the V encoded idiotypes, will not be included in the network. Therefore, the T cell would be susceptible to anti-idiotypic network signals only through its allelically excluded V-gene encoded receptor. One exception to the latter postulate might occur in allogeneic responses. Immunisation of an animal with allogeneic cells (MHC differences) will result in production of antibody to the MHC antigens. An antibody response to the R anti-H molecules on the antigenic cells could also occur. Antibody specific for R anti-H molecules might be expected to act like antibody against the idiomotype of anti-MHC antibodies⁴⁶ and therefore might regulate the production of antibody against MHC antigens.

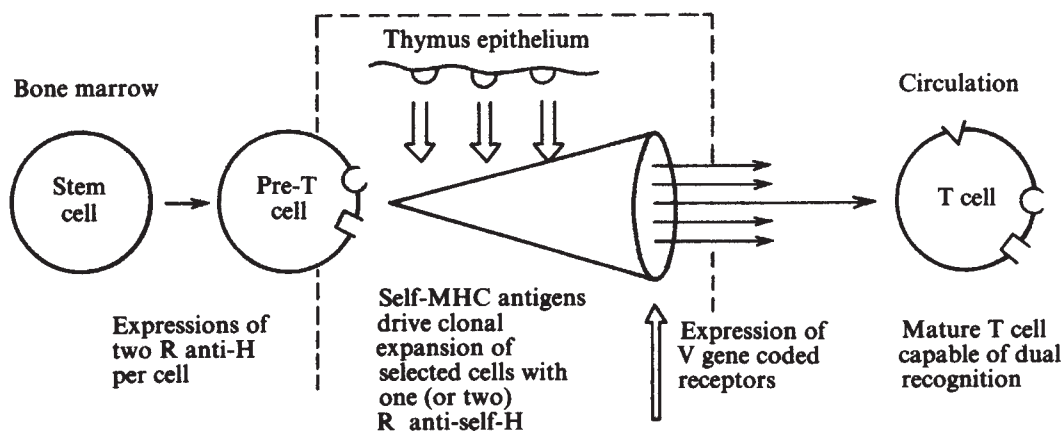
The reverse situation, that anti-idiotypic antibodies specific for the V encoded idiotypes cross react with R anti-H molecules, is also possible and might account for those data indicating a high percentage of T cells bearing allo-MHC specific idiotypes⁴⁷. Note that in other studies such a high percentage of idiomotype-bearing T cells has not been found⁴⁸.

The possibility, engendered by the present model, that antibodies might be raised against R anti-H in an immunisation regimen designed to raise antibodies against MHC antigens, has implications for the use of such antisera in functional assays. Blocking of T-cell function attributed to anti-MHC antibodies could be due to the presence of anti- (R anti-H) antibodies. Monoclonal anti-MHC antibodies will not be subject to this problem.

Function of T-cell receptors: responses involving K and D regions

This presentation is concerned mainly with recognition processes but must also relate the function of T-cell receptors. The

Fig. 2 Stages in T-lymphocyte maturation.



functional aspects of dual recognition have been discussed extensively by Langman⁴⁹ and by Cohn and Epstein¹⁸. The fact that the receptors in the present model arise in part from genes not previously invoked, and without the need for the somatic mutation and selection necessary in previous models does not alter the functional arguments. The requirement for a concerted response to X and an MHC antigen can be met either by interaction between X and MHC¹⁹ or by interaction between the receptors for X and MHC¹⁸. In the present model an interaction between the two types of receptor occurs and is necessarily selected for during development. This interaction prevents stimulation of the R anti-H receptor. Binding of antigen to V anti-X will make R anti-H available to bind H present on the same cell surface as X. When R anti-H binds H, this completes the stimulatory complex initiating the precursor cell to proliferate and differentiate and triggering the cytotoxic cell to kill. The stimulating signals can be delivered to the cell via either R anti-H alone or R anti-H plus V anti-X; this is true in the present model for all interactions, whether self presentation or allogeneic responses, because an R anti-H molecule is always involved. There is no need to postulate (as in other models¹⁸) either that no signal can be delivered by the R anti-H receptor or that allogeneic reactions do not involve the R anti-H receptor.

It is necessary to postulate that interaction of V anti-X with X while the R anti-H remains unsatisfied will not deliver a signal to the T cell. Indeed the requirement for both receptors to be filled for passive absorption of cytotoxic T cells⁵⁰ suggests that the V anti-X interaction with X may be transient.

Functions of T-cell receptors: responses involving the I region

A special role has been demonstrated for the I-region products in the generation and function of helper cells for antibody production^{4,5} or in mixed lymphocyte reactions (MLR)⁵¹. However, MLR stimulation can be demonstrated between cells matched at the MHC⁵² or differing at K or D antigens but matched for the I region; the extreme examples are the H-2 mutants which appear to be point mutations of either the K or D products⁵³. In the present model all MLR stimulation will involve dual recognition. In a fully allogeneic MLR, including antigenic differences at I-region gene products, dual recognition will involve an R anti-allo-I molecule and a V gene encoded receptor specific for any antigenic difference. With a minimal difference the active cells in MLR between parent strain and H-2 mutant strain would be T cells carrying R anti-self-I together with V receptors specific for the mutant histocompatibility antigens.

A valid model of T-cell function should explain MHC-linked immune response (*Ir*) genes⁵⁴. These *Ir* genes regulate the production of antibody. An *Ir* genotype is classified as allowing either high or low responsiveness; the low responder phenotype exhibits a lack of functional helper T cells specific for the antigen under *Ir* gene control (but low responders do have T cells

capable of binding that antigen⁵⁵). Inheritance of *Ir* genes has been interpreted as showing either dominance of the high responder gene⁵⁴ or co-dominance⁵⁶.

A recurrent explanation^{18,19,54} offered for *Ir* gene function is that *Ir* genes encode the T-cell receptor. Such explanations postulate that the structural genes for the T-cell receptor map in the I region, thus contradicting evidence that the T-cell receptor is encoded by structural genes linked to immunoglobulin heavy chain genes⁴³ (although there is evidence for genes at the MHC controlling T-cell receptor expression⁴⁸). Two contrasting explanations of the non-responder phenotype have been offered in terms of the somatic mutability of V genes. In one model¹⁵ all V anti-X genes are generated by mutation of V anti-self genes but in the *Ir* low responder a V gene specific for the antigen cannot be generated from any anti-self V gene. In another model^{18,49} it is postulated that only an anti-self V gene can generate a V gene coding for a product specific for the antigen under *Ir* gene control and low response is due to the need to retain V anti-self unmutated to perform its function in dual recognition. In neither of these two models is it obvious why *Ir* genes controlling antibody production map in the I region rather than in K and D regions of the murine MHC. A different explanation for *Ir* defects describes *Ir* gene products as a second recognition system with non-responsiveness resulting when the *Ir* gene product cannot associate effectively with an antigen¹⁹. The explanation for MHC-linked *Ir* genes in the present model attributes non-responsiveness to the binding of the antigen to the R anti-I specific for the non-responder I-region product. The functional *Ir* gene is thus one of the T-cell receptors; it is not encoded at a locus linked either to an immunoglobulin gene family or to MHC but expression of *Ir* gene phenotype will be controlled by I-region products so that the MHC appears to encode *Ir* genes.

Complete humoral responsiveness to every antigen is postulated to involve the R anti-I T-cell receptor but in most cases *Ir* control is not seen because low responsiveness is displayed only when an antigen fortuitously binds to the R anti-I, thus blocking the interaction of R anti-I with I in the dual recognition process. This defect will be seen at the level of antigen presentation on macrophages and at the level of co-operation between T and B lymphocytes because both processes involve simultaneous recognition of I region and antigen. The postulate that *Ir* phenotype coincides with R anti-I specificity leads automatically to an explanation for the findings that pre-T cells with a non-responder phenotype, when processed by a thymus of responder genotype, emerge as responder T cells⁵⁷ because there will have been selection for R anti-responder-I specificity. In keeping with the evidence⁵⁷, the model says that T cells generated thus will only function as responder helper cells when complementing responder B cells and macrophages. Because this model requires that all R anti-self-I molecules are selected for in the thymus it follows that *Ir* genes should show co-dominant expression⁵⁶.

Predictions

The model proposed here predicts that each T cell carries three receptor molecules. Two of these are non-antibody receptors, at least one of which is specific for a self-MHC molecule (either K, D or I in the mouse). Detection and characterisation of R anti-H molecules would provide evidence for the model. A small fraction of T-cell clones responding to a foreign antigen recognised together with a self-H antigen will also be able to recognise the antigen presented with an allo-H antigen due to the postulated third receptor (R anti-allo-H). Evidence for the latter subset of T-cell clones has been published³¹.

Receptors specific for self-MHC are postulated to be the products of a multigene family encoding receptors for many

allo-MHC products. The extent of that gene family will be revealed by further tests of the ability of pre-T cells to be selected in the thymus for recognition of a range of MHC types as self. Because expression of receptors for self-MHC molecules is postulated to be selected by the MHC antigens of the thymus the MHC appears to code for the T-cell receptor. An involvement of anti-self-MHC receptors in I-region linked non-responsiveness is postulated.

While recognising that I have invoked a new gene family that may or may not exist, I trust that the concept has some merit in ordering existing data and that it may influence future experimental design.

I thank Andrew McMichael, Dale Kipp and John Shire for stimulating and helpful discussion.

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ARTICLES

Observations of flexure and the geological evolution of the Pacific Ocean basin

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The response of the Pacific plate to oceanic island and seamount loads has been used to estimate the distribution of ridge crest and off-ridge volcanism on the plate since the late Jurassic. The most extensive event, forming the Hess rise, Line Islands ridge, Necker ridge, and Robbie ridge, occurred on the Pacific/Farallon and Pacific/Phoenix ridge crests during the interval 90-120 Myr BP (Barremian to Turonian). This event, which extended over a much larger area than comparable volcanism on the continents, is one of the largest to be documented in the geological record.

THE Pacific Ocean floor is characterised by numerous oceanic islands and seamounts of mainly volcanic origin¹⁻⁴. Although the age of the oceanic crust of the Pacific basin is now reasonably well known from marine magnetic lineations and Deep Sea Drilling Project (DSDP) sites, the origin and age of these volcanic features are largely unknown. These oceanic islands and seamounts are of geological interest as they are located within the interior of the Pacific plate and may therefore provide information on intra-plate volcanism. This article presents a method, based on oceanic plate flexure, for determining the

origin and age of some of these volcanic features in the Pacific Ocean basin.

Lithospheric flexure

The concept of plate tectonics is based on a strong rigid lithosphere which overlies a weaker asthenosphere. The principal evidence for a strong lithosphere on long time scales (>10⁶ yr) has come from studies of its response to surface loads⁵⁻¹⁵. These studies have shown that in several cases, geological and geo-

physical data in the region of surface loads can be explained by a simple model in which the lithosphere responds to long-term loads in a manner similar to that of a thin elastic plate overlying a weak fluid.

The results of recent studies of oceanic plate flexure are summarised in Fig. 1 in which the elastic thickness of the oceanic lithosphere T_e is plotted as a function of the age of the lithosphere at the time of loading. Different types of geological features have been plotted in Fig. 1. The age of the lithosphere at the time of loading was estimated at each feature by subtracting the age of the load from the age of the underlying sea floor. The studies by Watts⁹ and McNutt and Menard¹⁰ are shown as dashed lines because a single estimate of T_e was obtained for a wide range of age at the time of loading (>60 Myr). Also shown as a dashed line is the study by Caldwell¹⁴ at the Aleutian trench as it is difficult to separate the Aleutian outer rise from a broad regional rise in bathymetry which occurs seawards of the trench¹⁶. Figure 1 summarises all recent estimates of T_e , except those from the Philippine and Tonga-Kermadec trenches¹⁴ where the age of the subducting oceanic crust is too poorly known.

The main results shown in Fig. 1 are that surface loads formed on young lithosphere, such as the topography of oceanic Layer 2, are associated with relatively small values of T_e while loads formed on old lithosphere, such as the Hawaiian-Emperor seamount chain, are associated with relatively high values. The simplest interpretation of these results is that as the oceanic lithosphere increases in age it cools, thickens, and becomes more rigid in its response to surface loads. Figure 1 shows that there is good general agreement between T_e and the 300 °C and 600 °C oceanic isotherms, based on a cooling plate model¹⁷.

As the age of the surface loads plotted in Fig. 1 are in the range of a few Myr to several tens of Myr, Fig. 1 suggests T_e is acquired at the time of loading and does not change appreciably with time. This result is consistent with thermal models which predict that the thickness of the lithosphere increases with age⁹ and with inferences of the rheology of the oceanic lithosphere based on extrapolations of data from experimental rock mechanics^{18,19}.

The results in Fig. 1 have important implications for studies of the geological evolution of the ocean basins. For example, Fig. 1 suggests that an oceanic island or seamount formed on young sea floor would be associated with a relatively small T_e , while an oceanic island or seamount formed on old sea floor would be associated with a relatively large T_e . Thus by determining T_e at an oceanic island or seamount of unknown origin it should be possible to estimate whether it formed on young (ridge crest) or old sea floor (off-ridge).

A geophysical observation which can be relatively easily measured on a surface-ship and depends on T_e is the free-air gravity anomaly^{7-9,12}. For the theoretical seamount load shown in Fig. 2 there is a significant difference in both amplitude and wavelength between computed gravity anomaly profiles for $T_e = 5$ km, corresponding (Fig. 1) to formation of the load on relatively young sea floor (2–8 Myr), and $T_e = 25$ km, corresponding to formation on relatively old sea floor (>35 Myr). Thus since the overall accuracy of gravity data in the Pacific Ocean is 5–10 mGal (ref. 16), Fig. 2 suggests it should be possible to use observed free-air gravity anomaly profiles to distinguish whether a geological feature originated on a ridge crest or off-ridge.

Data analysis

We have compared observed free-air gravity anomaly profiles obtained on more than 100 cruises of mainly US and USSR research vessels in the Pacific Ocean to calculated profiles based on the elastic plate model. We assumed values of $T_e = 5$ and 25 km since these values clearly represent a ridge crest or off-ridge origin respectively (Fig. 1). The calculated profiles were obtained using linear transfer function techniques^{7,9} in which theoretical filters are constructed and convolved with observed bathymetry profiles. Both two- and three-dimensional

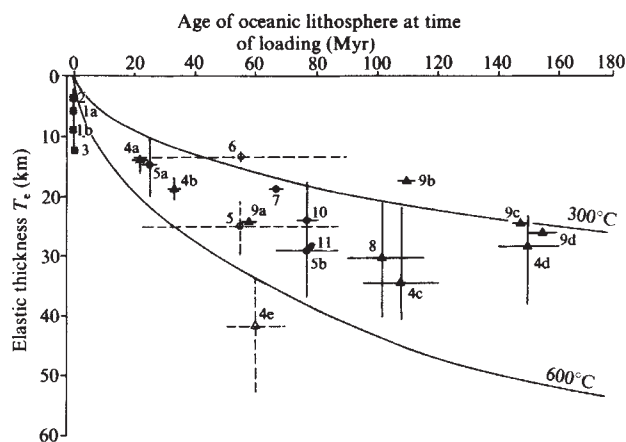


Fig. 1 Plot of elastic thickness of the oceanic lithosphere T_e against age of the lithosphere at the time of loading. 1a, East Pacific rise crest⁵; 1b, Mid-Atlantic Ridge crest⁵; 2, Juan da Fuca ridge crest⁶; 3, Mid-Atlantic Ridge crest⁷; 4, Deep-sea trench outer rise systems¹⁴; 4a, Nankai trough; 4b, Middle America trench; 4c, Kuril trench; 4d, Mariana trench; 4e, Aleutian trench; 5, Hawaiian-Emperor seamount chain⁹; 5a, Emperor seamounts north of 40° N; 5b, Hawaiian ridge and Emperor seamounts south of 40° N; 6, Pacific Ocean atolls¹⁰ (Cook Islands, Australs, Tuamotu Islands and Henderson); 7, Great Meteor seamount, NE Atlantic⁸; 8, Kuril trench¹⁵; 9, Deep sea trench-outer rise systems¹³; 9a, Aleutian trench; 9b, Kuril trench; 9c, Bonin trench; 9d, Mariana trench; 10, Hawaiian ridge¹¹; 11, Hawaii Island¹². Each estimate of T_e is related to the flexural rigidity of the plate and is based on an assumed Young's modulus of 1×10^{12} dyn cm⁻². The age of the lithosphere at the time of loading was estimated by subtracting the age of each load from the age of the underlying sea floor. For the ridge crest estimates and trench estimates the loading is assumed to be of the present age (0 Myr). For the seamount and oceanic island estimates, the age of loading is obtained from dated samples on the crests or flanks of these features. The solid lines are the 300 °C and 600 °C oceanic isotherms based on a cooling plate model¹⁷.

filters⁷ and the effects of non-linearity in the computation of the gravity anomaly have been considered.

Figure 3 shows the comparison between observed and computed gravity anomaly profiles for four selected geological features in the Pacific Ocean. The observed gravity anomaly profiles of the Line Islands ridge and Mid-Pacific mountains can be most satisfactorily explained by the computed profile with $T_e = 5$ km (Fig. 3a). The computed profile with $T_e = 25$ km predicts too large an amplitude gravity anomaly over the crest of these features compared with the observed amplitude and wavelengths that are too long over flanking regions. The observed gravity anomaly profiles of Makarov Guyot³ and Louisville ridge, however, can be most satisfactorily explained by the computed profile with $T_e = 25$ km (Fig. 3b). The computed profile with $T_e = 5$ km predicts too small an amplitude over the crest of these features compared with the observed amplitude and wavelengths that are too short over flanking regions.

The comparisons in Fig. 3 can therefore be interpreted as indicating the Line islands ridge and Mid-Pacific mountains formed on a ridge crest (on sea floor of 2–8 Myr age) while Makarov Guyot and Louisville ridge formed off-ridge (on sea floor of >35 Myr age). These conclusions on the origin of these features are, of course, uncertain as they are based on a single gravity anomaly and bathymetry profile and refer only to the origin of the major part of each feature. We have shown²¹, however, that uncertainties in the computed profiles, due either to variations in the crustal structure and density of topography assumed or to non-linearity in the computed gravity effects would not significantly alter these conclusions.

Results

The results of the data analysis for each of the cruises in the Pacific Ocean are summarised in Fig. 4: the symbols represent an estimate of the geological setting of an individual bathymetric feature along each ship track. Figure 4a summarises those

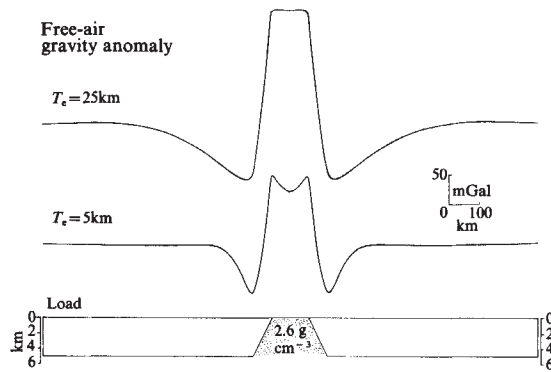


Fig. 2 Computed free-air gravity anomaly profiles based on the continuous elastic plate model and the theoretical seamount load shown. The flexure of the plate has been computed using linear theory²⁰ and the free-air gravity anomaly has been computed using a two-dimensional line-integral method.

estimates which are interpreted to be of ridge crest origin and Fig. 4b those estimates which are of off-ridge origin.

A comparison of Fig. 4a with available bathymetry maps of the Pacific Ocean^{24,25} show that most of the ridge crest estimates correlate with bathymetric features in the western and central Pacific. These include the Manihiki plateau, Shatsky rise, Hess rise, Necker ridge, Line Islands ridge and Tuamotu Islands and some features in the Magellan seamounts, Mid-Pacific mountains and Emperor seamounts. A number of these features were associated with more than two ridge crest estimates (for example, Shatsky rise (5), Necker ridge (3), Hess rise (4), Line Islands ridge (5) and Tuamotu Islands (10)). Most of the ridge crest estimates (Fig. 4a) apparently occur between the 90 and 120 Myr isochrons, based on magnetic anomaly lineations and DSDP sites²².

The off-ridge estimates (Fig. 4b) mainly correlate with bathymetric features in the western Pacific Ocean. These include Society Islands, Samoa, Melanesian border plateau²³, Cross-Line Island trend and Louisville ridge and some features of the Hawaiian ridge and Emperor seamounts, Geisha and Marcus-Wake Guyots³ and Magellan seamounts. A number of these features were associated with more than two off-ridge estimates (for example, Samoa (3), unnamed seamount in the Magellan seamounts (3), Louisville ridge (3), and Society Islands (3)). The off-ridge estimates show no obvious relationship to the isochrons although the estimates for the Cross-Line Island trend, Hawaiian ridge and Marcus-Wake Guyots correlate with bathymetric features which generally trend WNW-ESE⁴, across the local trend of the isochrons.

We recognise that although data from a large number of cruises have been used in this study, the number of estimates in Fig. 4 is small compared with the total number of oceanic islands and seamounts which actually exist in the Pacific Ocean. We believe, however, that although any one estimate in Fig. 4 may be subject to uncertainties, the occurrence of several similar estimates from the same geological feature is evidence that the overall distribution of ridge crest and off-ridge estimates (Fig. 4) is correct. A better test of their reliability, however, is to compare the predicted age of the bathymetric features associated with these estimates to geological ages based on bottom sampling.

The ages of the bathymetric features associated with each estimate (Fig. 4) can be predicted if the age of the underlying sea floor is known (Fig. 1). Table 1 summarises those estimates which are associated with a nearby reliably dated bottom sample. There is a good general agreement between predicted and geological ages for the ridge crest estimates, particularly for Hodgkins, Line Islands, Easter Island and the Tuamotu Islands. The agreement is not as good for the off-ridge estimates because the predicted ages are only maximum estimates.

Table 1 therefore suggests that it should be possible, at least for the ridge crest estimates (Fig. 4a), to predict the age of the

associated bathymetric features with some confidence. The main uncertainties, however, are in estimating the age of the sea floor, particularly in regions of the magnetic quiet zones. We tabulated the predicted ages for each ridge crest estimate in 30-Myr intervals through geological time. The smallest number of estimates (3) occur for the interval 150–180 Myr BP (Kimmeridgian to Pliensbachian) and the largest number of estimates (26) occur for the interval 90–120 Myr BP (Barremian to Turonian).

The distribution of the off-ridge and ridge crest estimates in the Pacific Ocean basin for the 90–120 Myr BP interval are plotted in Fig. 5. The solid triangles represent ridge crest estimates associated with the Hess rise, Necker ridge, Line Islands ridge, Manihiki plateau and Robbie ridge and the solid circles represent off-ridge estimates associated with the Geisha Guyots, for which there is geological control (Table 1). The bathymetric features associated with the ridge crest estimates extend over a broad region of the Pacific Ocean basin (Fig. 5). We therefore infer that the interval 90–120 Myr BP was a time of significant volcanism on the Pacific plate.

As the solid triangles in Fig. 5 represent ridge crest estimates they may indicate the former position of the accreting Pacific

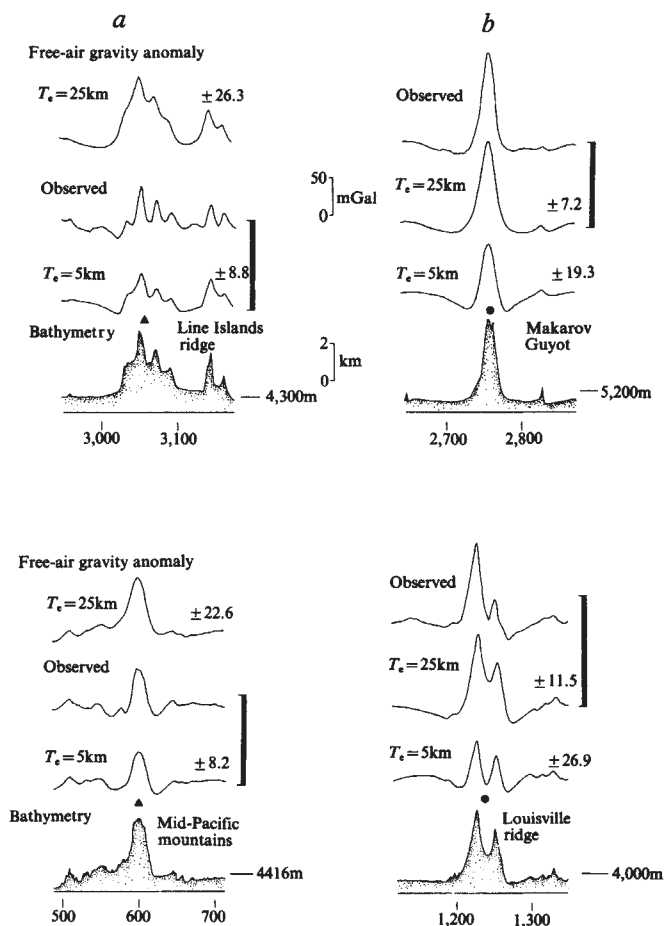


Fig. 3 Comparison of observed free-air gravity anomaly profiles with computed profiles based on the elastic plate model and assumed values of the elastic thickness $T_e = 5$ km and 25 km. *a*, USNS Eitanin 31 profile of the Line Islands ridge at lat 1.7° N and long 157.0° W and RV Vema 24 profile of the Mid-Pacific mountains at lat 18.5° N and long 179.5° W. *b*, RV Vema 21 profile of Makarov Guyot at lat 29.6° S and long 153.5° E and USNS Eitanin 40 profile of Louisville ridge at lat 39.3° S and long 167.5° W. The observed profiles of the Line Islands ridge and Mid-Pacific mountains can be best explained by the computed profile of $T_e = 5$ km and the observed profiles of Makarov Guyot and Louisville ridge can be best explained by the profile for $T_e = 25$ km. A ridge crest origin ($\blacktriangle$) has been assigned to the Line Islands ridge and Mid-Pacific mountains; an off-ridge origin ($\bullet$) has been assigned to Makarov Guyot and Louisville ridge. The numbers to the right of each computed profile are the r.m.s. differences between observed and calculated gravity anomalies and the number below each bathymetry profile is the distance in nautical miles along a ship track, used to locate each feature.

Table 1 Comparison of predicted ages based on flexure studies with geological ages

Seamount or oceanic island	Ship crossing feature	Position of ship crossing		Method of geological age determination	Position of geological age		Estimated age of underlying sea floor (Myr BP)	Predicted (flexure) age* (Myr BP)	Geological age (Myr BP)	Geological age ref.
		Lat	Long		Lat	Long				
Ridge Crest										
Hodgkins (Kodiak-Bowie)	Conrad	53.3	-135.6	K-Ar	53.5	-136.0	17	12 ± 3	14-15	26
Necker ridge	Vema	22.5	-166.7	K-Ar	21.5	-167.9	100	95 ± 3	>61	27
Line Islands	Kana Keoki	3.8	-159.4	DSDP Site 315	4.2	-158.5	102	97 ± 3	91 ± 3	28
Shepard Guyot	Vema	18.5	-179.5	Forams	19.2	-179.5	137	132 ± 3	≥Cen-Tur.	3
Manihiki plateau	Kana Keoki	-12.1	-160.9	DSDP Site 317	-11.0	-162.0	290	285 ± 3	≥105	28
Shatsky rise	Conrad	32.1	155.1	DSDP Sites 305, 306	32.0	157.8	136	131 ± 3	≥120	29
Easter Island	Conrad	-27.2	-109.5	K-Ar	Samples from island		4	<2	3	29
Tuamotu	Conrad	-15.6	-147.4	DSDP Site 318	-14.8	-146.8	61	56 ± 3	49-53	30
Wentworth	Kana Keoki	28.8	-177.9	K-Ar	28.8	-177.8	110	105 ± 3	≥71	31
Off-ridge										
Isakov Guyot	Vema	31.6	151.1	Fossil	31.6	151.2	148	<113	≥Lower cretaceous	3
Makarov Guyot	Vema	29.5	153.5	Fossil	29.5	153.3	155	<120	≥Cen-Tur.	3
Z4/3 Guyot†	Vema	27.1	148.7	K-Ar	27.1	148.7	154	<120	≥86-96	32
Lamont Guyot	Argo	21.7	159.4	Fossil	21.5	159.2	170	<135	≥Mid-Eocene	3
Ita Mai Tai Guyot	Dmitri	12.9	157.0	DSDP sites 200, 202	12.8	156.8	171	<136	≥Early Eocene	33
Somoa (Savaii)	Dmitri	-13.3	-172.3	Historic eruption	-13.6	-172.4	107	<72	Historic eruption	34
Society	Kana Keoki	-16.1	-151.3	K-Ar	-16.7	-151.0	71	<36	1.9-5.4	35

* Computed assuming a ridge crest estimate corresponds to an age of the lithosphere at the time of loading of 2-8 Myr and an off-ridge estimate corresponds to an age of >35 Myr.

† This seamount has a reliable palaeomagnetic pole⁴⁹. The new constraints on the age suggests this pole (lat. 53.0° N, long. 302°) is probably of early Cretaceous age.

plate boundary. In particular, the Line Islands ridge, Necker ridge and Hess rise probably formed close to the crest of the former Pacific/Farallon plate boundary³⁶ and the Robbie ridge probably formed close to the crest of the former Pacific/Phoenix plate boundary³⁶. Therefore, either volcanism occurred along a major portion of the Pacific plate boundary during 90-120 Myr BP or, beginning about 90-100 Myr BP major ridge jumps occurred along the Pacific plate boundary⁴ accompanied by extensive volcanism on the abandoned ridges. Because of the scatter of the ridge crest estimates (Fig. 5) and the fact that only one limb of the 90-120 Myr BP spreading system is preserved at present, it is not possible to distinguish between these possibilities.

Recent evidence from DSDP site 462³⁷ and from seismic reflection profiling with a large volume sound source³⁸ support the earlier suggestion of Winterer⁴ that the relatively old crust of the central Pacific basin and the Line Islands area may be obscured by a younger deep-water volcanic event. DSDP site 462³⁷ (Fig. 5), drilled on oceanic crust of probable late Jurassic age, recovered a volcanic igneous complex of Albian to Aptian age (100-110 Myr BP). Houtz and Ludwig³⁸ have mapped a seismic reverberant layer, using sonobuoy and single channel vertical incidence data, which reaches thicknesses exceeding 2 km in parts of the central Pacific Ocean basin. Although the reverberant layer may be of calcareous origin³⁸, the presence of a thick layer (>600 m) in the region of DSDP sites 462 and 169 suggests the layer may be, in part, of volcanic origin and therefore similar in age to the volcanic rocks drilled at the DSDP sites.

These considerations suggest the 90-120 Myr BP ridge crest volcanism which formed the Line Islands ridge, Hess rise and Necker ridge (Fig. 5) may have been accompanied by an extensive deep-water volcanic event in the adjacent ocean basins. The solid line in Fig. 5 outlines the area of the Pacific plate which seems to have been most affected by this event. We have included only evidence from the ridge crest estimates (Fig. 4a), the off-ridge estimates for which there is geological control (Table 1) and the contemporaneous deep-water volcanism inferred from DSDP sites and seismic reflection profiling. Thus although other undated oceanic islands and seamounts in the Pacific, not crossed by the cruises, may possibly be of similar age to this event we have not plotted them in Fig. 5.

Discussion

We have documented a major geological event involving an extensive outpouring of ridge crest volcanism on the Pacific plate during the interval 90-120 Myr BP (Barremian to Turonian). This event was probably accompanied by deep-water volcanism. The area of the plate affected by the volcanism is ~10⁷ km² (Fig. 5), which is significantly larger than areas of comparable volcanism on the continents (for example, western US since the Miocene³⁹).

The origin of the ridge crest volcanism on the Pacific plate during the interval 90-120 Myr BP is not clear at present. The fixed or moving hot-spot hypothesis⁴⁰⁻⁴² cannot simply explain the volcanism unless a large number of contemporaneous hot spots are proposed along or near the former Pacific/Farallon and Pacific/Phoenix plate boundaries. The lithospheric fracture hypothesis⁴³ explains the widespread similarity in ages but cannot explain the volcanism unless a stress system is invoked which causes tensional failure of the plate in a direction generally parallel to the trend of the former Pacific/Farallon and Pacific/Phoenix plate boundaries. Thermal contraction of the oceanic plate⁴⁴, for example, would cause tensional failure which propagates in the direction of sea floor spreading.

An additional source of stress in the plates, however, are those associated with the driving mechanism of plate tectonics⁴⁵. There is evidence, for example, from magnetic lineation patterns³⁶ that the 90-120 Myr BP volcanic event on the Pacific plate (Fig. 5) occurred at a time of relatively rapid spreading rate on the Pacific/Farallon ridge crest. This spreading rate increase, if it occurred⁴⁶, may have been accompanied by either the initiation of subduction or an increase in the convergence rate on another Pacific plate boundary. If this increase in convergence rate was accompanied by an increase in the driving force on the subducting plate⁴⁷, additional stresses may have been transmitted to the horizontal part of the plate. These stresses may have been sufficient to cause the Pacific plate to fracture at its weakest point and allow magma to flow from the underlying asthenosphere to the area around the ridge crest.

We have also documented the distribution of off-ridge volcanism on the Pacific plate (Fig. 4b). As some of the estimates of off-ridge volcanism are from the oldest part of the Pacific plate (Fig. 4b) this volcanism is therefore of late Jurassic or younger age. The main problem, however, is that with the exception of

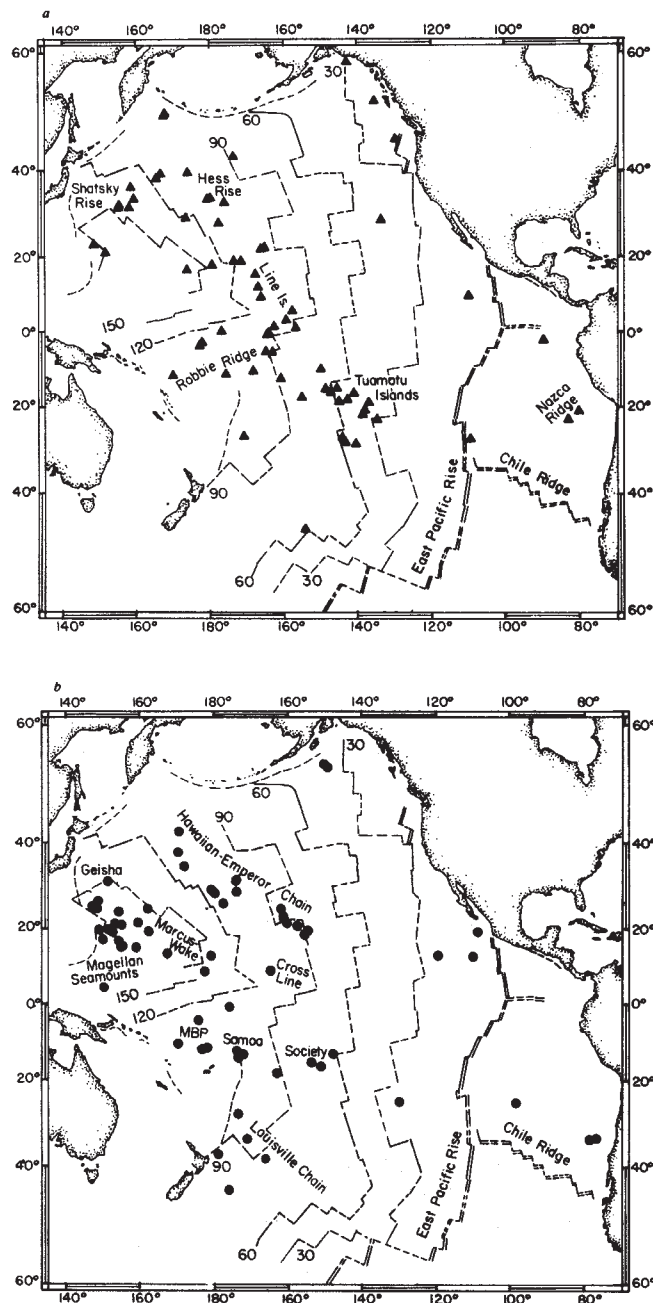


Fig. 4 Plot of estimates of ridge crest (a) and off-ridge volcanism (b) on the Pacific plate. The solid lines indicate isochrons based on observed magnetic lineations and DSDP sites and the dashed lines indicate interpolated isochrons based on tectonic reconstructions for the Pacific plate²². MBP, Melanesian border plateau²³.

the Hawaiian-Emperor seamounts and some features in the Geisha Guyots, where there is adequate geological control, the age of the off-ridge volcanism is generally unknown (for example, Table 1).

There is evidence, however, that at least part of the off-ridge volcanism in Fig. 4b may be of younger age than 90–120 Myr BP. The Line Islands ridge between latitudes 5°N and 15°N are intersected by the WNW-ESE Cross-Line Island trend which, based on bathymetry⁴ and the estimated age of the off-ridge estimate at latitude 12°N longitude 165°W (Fig. 4b), are younger than the Line Islands ridge. The Melanesian border plateau, Samoa and Society Islands, which seem to form part of a broad WNW-ESE trend in off-ridge volcanism in the central Pacific (Fig. 4b), are probably younger than middle Cretaceous

(see Table 1). The age of the off-ridge volcanism of the Magellan seamounts and Marcus-Wake Guyots is not known, however, although they are associated with similar WNW-ESE trends in bathymetry as the Cross-Line Island trend^{4,24}.

The presence of WNW-ESE trends in the bathymetry of features associated with off-ridge volcanism (Marcus-Wake Guyots, Magellan seamounts, Cross-Line Island trend) is of particular interest as they cross isochrons and are generally parallel to the direction of present day absolute Pacific plate motions. One possibility therefore is that the off-ridge volcanism

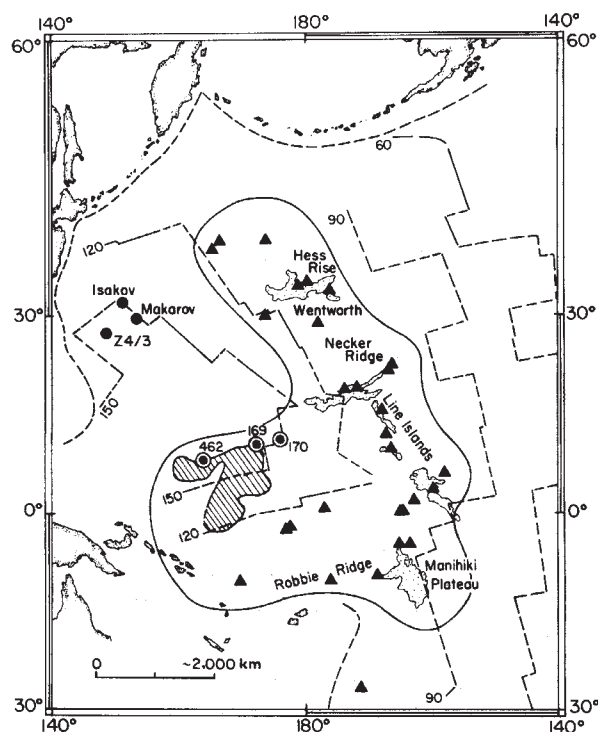


Fig. 5 Map summarizing the inferred distribution of volcanism on the Pacific plate during the interval 90–120 Myr BP (Barremian to Turonian). ▲, Bathymetric features formed at a ridge crest; ●, features formed off-ridge. The ridge crest features probably originated on or near the Pacific/Farallon and Pacific/Phoenix ridge crests³⁶ and the off-ridge features originated in the interior of the Pacific plate away from the accreting plate boundaries. ◎, The deep water DSDP sites which were drilled on oceanic crust of probable late Jurassic age and sampled basaltic igneous rocks of Albian to Aptian age (100–110 Myr BP). The shaded area is the >600-m isopach of the seismic reverberant layer³⁸ in the region of DSDP sites 462 and 169 and the stippled area indicates the extent of shallow (<4,000 m)²⁴ sea floor in the region of the Manihiki plateau, Line Islands ridge, Necker ridge, Hess rise and Mid-Pacific mountains.

is a surface expression of small-scale convection⁴⁸ in the underlying mantle. The ages of these features are not well enough known, however, and more studies need to be carried out before this possibility can be examined.

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In vitro and *in vivo* products of *E. coli* lactose permease gene are identical

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The lacY gene product synthesised in vitro is identical to lactose permease isolated from cytoplasmic membranes as determined by apparent molecular weight and N-terminal amino acid sequence. The amino acid composition of the in vivo product agrees well with that predicted from the DNA sequence. The data assign the translational start on the DNA sequence and demonstrate that this protein is processed only by deformylation but not by proteolytic cleavage at the N-terminus.

IN both bacterial and eukaryotic cells, most secreted proteins are initially synthesised as precursors with hydrophobic amino-terminal extensions which are removed by proteolytic cleavage during or after translocation of the protein through the membrane^{1,2}. Such precursors have been demonstrated for some integral membrane proteins, such as the coat protein of the bacteriophage M13 (refs 3, 4), whereas in other cases the primary translation product formed *in vitro* seems to be identical to the membrane-associated *in vivo* product^{5,6}. We describe here the *in vitro* synthesis of lactose permease (lactose carrier, product of the *Y* gene of the *lac* operon) of *Escherichia coli*⁷ and show that the *in vitro* product is identical to permease isolated from the cytoplasmic membrane of this organism. As a model for the biosynthesis of a hydrophobic protein, lactose permease seems particularly attractive because this protein must attain a highly organised tertiary structure in the membrane in order to function as a lactose/proton-symporter⁸.

Cell-free protein synthesis directed by hybrid plasmid pGM21

pGM21 is a hybrid plasmid between the vector pACYC 184 (ref. 9) and a previously described *lac Y* gene-containing fragment^{10,11} integrated into the single site for the restriction enzyme *EcoRI* located in the gene conferring resistance to chloramphenicol. The fragment comprises about 2,300 base pairs and carries an intact *lac* promoter and operator region, an intact *Y* gene, but only minor parts of the *A* (thiogalactoside transacetylase), *Z* (β -galactosidase) and *I* (repressor) genes [genotype *lac* Δ (*I*)*P*⁺*O*⁺ Δ (*Z*)*Y*⁺ Δ (*A*)]. The restriction maps of this fragment and the *lac Y* gene-containing fragment sequenced in the preceding paper¹² are identical in the region of the *Y* gene. *In vivo*, pGM21-directed synthesis of lactose permease is inducible. The protein comprises up to 15% of the

cytoplasmic membrane protein and is functionally intact as judged by transport and substrate binding experiments^{10,13,14}.

When added to a coupled transcription-translation system according to Zubay *et al.*¹⁵, plasmid pGM21 efficiently promotes cell-free protein synthesis. In an incubation mixture containing ¹⁴C-phenylalanine as the only radioactive amino acid, 11% of the label is found in a high molecular weight (MW) form, whereas in the absence of pGM21 only 0.1% of the input radioactivity becomes acid precipitable. An aliquot of the total incubation mixture was analysed on a polyacrylamide gel (Fig. 1a). It is clear that pGM21 directs the synthesis of one major product (slice nos 36–42, 33% of radioactivity) of apparent MW 30,000 $\pm$ 1,000. This radioactive peak corresponds in both mobility and width to purified lactose permease (prominently stained band in gel at the top of Fig. 1) or permease labelled with ³H-N-ethylmaleimide in intact cytoplasmic membranes (Fig. 1b). Thus, the main product of the *in vitro* synthesis is identical to lactose permease within the resolution of the method.

As pGM21 contains an intact promoter-operator region, *in vitro* expression of the *Y* gene should be stimulated by cyclic AMP and reduced by *lac* repressor. Furthermore, repression should be relieved by addition of the inducer isopropyl β -D-thiogalactopyranoside (IPTG). Analysis of *in vitro* protein synthesis reaction mixtures on acrylamide gels demonstrates that the major product (apparent MW 30,000) is indeed subject to regulation by cyclic AMP and repressor (compare lanes 6–9 of Fig. 2). In terms of total incorporation of radioactive amino acids into protein (see Fig. 2 legend), cyclic AMP stimulates about twofold, repressor suppresses this synthesis by 60%, and addition of IPTG relieves the inhibition almost completely. These effects of cyclic AMP and repressor are small compared with control studies on *in vitro* synthesis of β -galactosidase directed by DNA from *lac* transducing phage λ in the same conditions. As quantitated by catalytic activity, the synthesis of this enzyme

was reduced to less than 5% of the control by the omission of cyclic AMP or by addition of repressor (data not shown). The different behaviour of the two DNA preparations may be related to the use of circular DNA in the case of pGM21.

Depending on the conditions of gel electrophoresis, variable amounts of radioactivity in polypeptide material are distributed almost homogeneously throughout the high-MW region of the gel. As lactose permease has a marked tendency to aggregate, this material could at least in part represent *Y* gene product. This assumption is supported by the observation that the diffusely distributed radioactivity is also subject to regulation by cyclic AMP and repressor (Fig. 2). Moreover, co-electrophoresis of ^3H -labelled protein synthesised in the presence of repressor and IPTG and ^{14}C -labelled protein made in the presence of repressor only shows that the normalised radioactivity profiles essentially coincide from the start to the front of the gel (data not shown). Thus, repressor affects not only the synthesis of the main product (apparent MW 30,000) but also that of material of lower mobility and small polypeptides moving to the front. The diffusely distributed radioactivity in the high-MW region together with the permease band form about 60% of the total radioactivity on the gel.

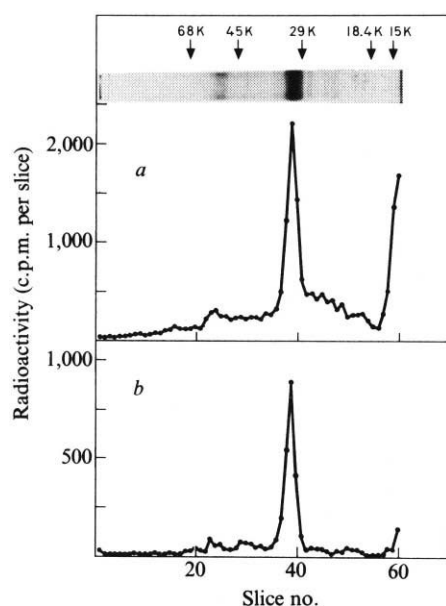


Fig. 1 Comparison of *in vivo* and *in vitro* *lac Y* gene products. *a*, Polyacrylamide-gel electrophoresis of *in vitro* products. A cell-free extract of *E. coli* K12 strain 514 (carrying a deletion of the *lac* operon) was used in a coupled transcription translation system as described previously^{15,19}. First, the extract was incubated for 60 min at 0°C with 1 mM phenylmethanesulphonyl fluoride. To minimise accumulation of degradation products and of incomplete polypeptides, radiolabelling by ^{14}C -phenylalanine was limited to the interval from 15 to 25 min after the start of the reaction and was followed by a 5-min chase with a 25-fold molar excess of unlabelled phenylalanine. The 20 unlabelled amino acids were present at 0.22 mM each, pGM21 DNA at 30 $\mu\text{g ml}^{-1}$ and polyethylene glycol 6000 at 25 mg ml^{-1} (all final concentrations). After the addition of ^{14}C -phenylalanine to 100 $\mu\text{Ci ml}^{-1}$, the concentration of this amino acid was 0.36 mM. A further incubation for 10 min at 37°C with DNase I and RNase A (each added to a final concentration of 25 $\mu\text{g ml}^{-1}$) terminated the reaction. The complete reaction mixture incorporated 10.5% of the ^{14}C -phenylalanine into material insoluble in hot trichloroacetic acid. In the absence of pGM21 DNA, only 0.1% of the label was incorporated. An aliquot of 3 μl synthesis mixture was mixed with 27 μl sample buffer, incubated for 30 min at room temperature and applied to a slab gel (compare with ref. 20, 14% acrylamide-0.12% bisacrylamide). Free radioactive phenylalanine was removed during staining with Coomassie brilliant blue R 250. The figure shows the radioactivity distribution in sections of the gel counted as described previously¹³. *b*, Radioactivity distribution of a cytoplasmic membrane preparation in which permease was labelled with ^3H -*N*-ethylmaleimide^{13,21}. The top of the figure shows electrophoresis of lactose permease isolated in an inactive state from strain T206 (ref. 16). Arrows refer to marker proteins (bovine serum albumin, MW 68,000; ovalbumin, 45,000; carbonic anhydrase, 29,000; β -lactalbumin, 18,400; haemoglobin, 15,000).

Table 1 Amino acid composition of *lac* permease

Amino acid	No. of residues
Asp	20.96 (6)
Asn	(16)
Thr	19.10 (19)
Ser	27.20 (29)
Glu	21.79 (11)
Gln	(11)
Pro	11.65 (12)
Gly	35.28 (36)
Ala	34.35 (35)
Cys	6.55 (8)
Val	28.70 (29)
Met	13.50 (14)
Ile	28.67 (33)
Leu	49.84 (54)
Tyr	11.67 (14)
Phe	35.40 (56)
His	4.28 (4)
Lys	12.05 (12)
Arg	12.56 (12)
Trp	4.72 (6)
Sum	(417)

Amino acids were analysed as described elsewhere²² on a Beckman 121M amino acid analyser. The numbers given for valine, isoleucine, leucine and phenylalanine were derived from 192-h hydrolysates. Cysteine was analysed as cysteic acid. The integral values in parentheses are based on the DNA sequence¹².

A control experiment is shown in Fig. 2 (lanes 3, 4). The vector pACYC184 codes for one major product concentrated in a sharp band of MW 25,000 $\pm$ 2,000. We attribute this band to the expression of the chloramphenicol resistance gene⁹ which is inactivated by insertion of the *lacY*-containing fragment. Therefore, this product is lacking in pGM21-directed *in vitro* synthesis.

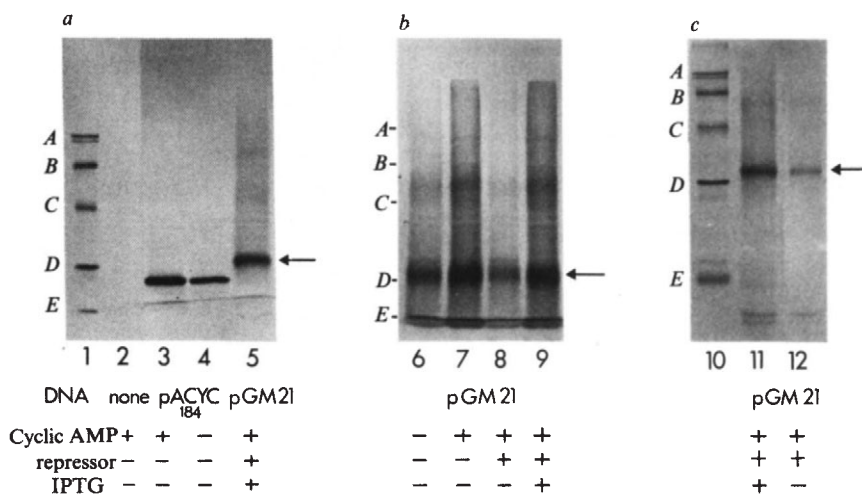
Amino-terminal sequence of the *lacY* gene product synthesised *in vitro*

The amino acid sequence of the product encoded by pGM21 DNA *in vitro* was partially elucidated. The material synthesised in the presence of ^{14}C -phenylalanine, ^3H -lysine, ^{14}C -leucine or ^{35}S -methionine was subjected to automated sequence analysis (Fig. 3). These four amino acids appear in the following positions—Phe: 9, 12, 15, 16, 17, 18, 20, 21, 27, 29 and 30; Lys: 5 and 42; Leu: 4, 14 and 34; Met: 1, 11 and 23. These assignments agree completely with the prediction of the DNA sequence¹² and thus identify the major protein synthesised from pGM21 DNA *in vitro* as the product of the *lacY* gene. This species represents the predominant product of cell-free synthesis, as sequence analysis does not reveal the presence of amino acids in positions other than those predicted by the DNA sequence.

Neither the permease nor another protein component exists predominantly with a formylated N-terminus. The ^{35}S -methionine liberated after the first degradation cycle is 74% of that observed when the product is chemically deformylated (Fig. 3d). The newly deblocked material contains no additional methionine residues up to position 21, suggesting that no different sequences are made available for analysis. Thus, methionine-1 is probably encoded by the initiation codon of the *Y* gene, and the protein is efficiently deformylated in the cell-free extract. The yield of radioactive methionine released from the deformylated material in the first sequencing step was 72% of that expected from the methionine content of permease (see Table 1 and Fig. 3). The yields of the more stable amino acids Phe, Leu and Lys were 82–91% (Fig. 3).

The *lac* permease synthesised *in vitro* was biosynthetically labelled with 15 amino acids and submitted to direct microsequence analysis (Fig. 4). After 42 sequencing steps, 34 residues could be identified. The unassigned positions correspond to

Fig. 2 Regulation of expression of pGM21 *in vitro*. DNA-directed cell-free protein synthesis was carried out as described for Fig. 1 with the following alterations. Radiolabelling for samples of *a* and *b* was continuous throughout a 30-min synthesis period with a mixture of ^{14}C -labelled amino acids (algal protein hydrolysate, Amersham-Buchler) added to the complement of 20 nonradioactive amino acids all at 0.2 mM final concentration. Additions indicated below the figure were as follows: pACYC184 (vector) or pGM21 (plasmid carrying *lacY*) DNA ($30\ \mu\text{g ml}^{-1}$), cyclic AMP (1 mM), *lac* repressor purified to the final DEAE-cellulose step (ref. 22, $8.5\ \mu\text{g ml}^{-1}$ for samples in *b* and $30\ \mu\text{g ml}^{-1}$ for samples in *c*), and IPTG (0.2 mM). Incorporation of radioactivity into hot trichloroacetic acid-insoluble material was 1, 13, 5 and 18% for lanes 2–5; 11, 24, 9 and 22% for lanes 6–9 and 10 and 3% for lanes 11 and 12, respectively. Aliquots of 5 μl (*a* and *b*) or 2.5 μl (*c*) were applied to 12% (*a* and *b*) or 20% (*c*) polyacrylamide gels without (*b*) or with (*a* and *c*) prior heating for 5 min to 95°C . ^{14}C -methylated marker proteins (A–E lanes 1 and 10): phosphorylase (MW 92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000) and cytochrome *c* (13,000).



the amino acids asparagine, histidine, tryptophan and serine, according to the DNA sequence. With the exception of serine, these amino acids were not present in the mixture of radioactive amino acids used for the labelling *in vitro*. The partial sequence obtained is: $\text{H}_2\text{N-Met-Tyr-Tyr-Leu-Lys-X-Thr-X-Phe-X-Met-Phe-Gly-Leu-Phe-Phe-Phe-Phe-Tyr-Phe-Phe-Ile-Met-Gly-Ala-Tyr-Phe-Pro-Phe-Phe-Pro-Ile-X-Leu-X-Asp-Ile-X-X-Ile-X-Lys}$ (42 residues; X, not determined). These residues correspond exactly to those predicted by the DNA sequence.

The ability to determine a unique sequence directly from the reaction mixture underscores the homogeneity of the *in vitro* product and the usefulness of pACYC184 as a vector provided only one gene is expressed from an insert into the *EcoRI* site.

Comparison of *in vivo* and *in vitro* products

lac permease isolated from the cytoplasmic membrane of the plasmid-containing *E. coli* strain T206 in an inactive state (see gel on top of Fig. 1 (ref. 16)) was degraded manually without prior chemical deformylation. Residues 1–9 and 11–15 of the *in*

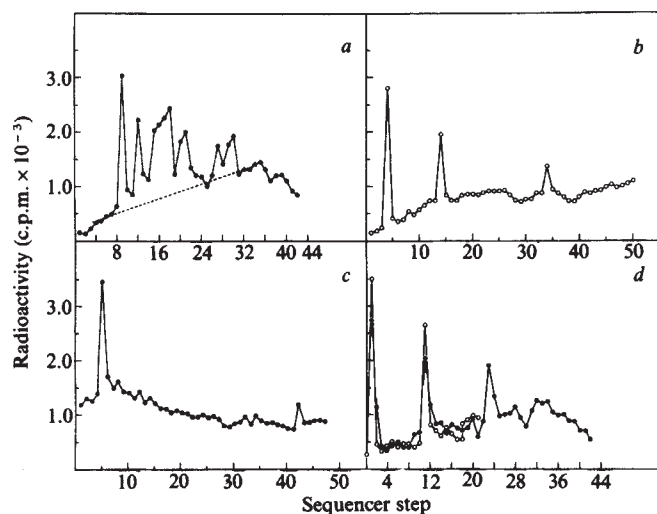


Fig. 3 Amino acid sequence analysis of the total cell-free products programmed by pGM21 plasmid DNA. The products were labelled with one radioactive amino acid at a time: *a*, ^{14}C -phenylalanine (256,000 c.p.m. in the sample analysed); *b*, ^{14}C -leucine (231,000 c.p.m.); *c*, ^3H -lysine (62,000 c.p.m.); *d*, ^{35}S -methionine (110,000 c.p.m. in both experiments). Total synthesis mixtures were dialysed against 50 mM ammonium bicarbonate, pH 8.5, lyophilised, chemically deformylated²³ (with the exception of *d*, ●), dialysed against 10% formic acid in the presence of 4 mg oxidised chicken lysozyme and subjected to automated Edman degradation in an updated Beckman model 890B sequencer equipped with a Sequemat P-6 automatic converter. Degradations were carried out in the presence of precycled polybrene (1.5 mg) using a 0.2 M quadrol program with double cleavage²⁴. The radioactivity in the fraction obtained at each cycle of degradation was determined in 10 ml Aquasol-2 (NEN) containing 2% methanol with a Packard Tri-Carb liquid scintillation spectrometer. The analysis of the phenyl thiohydantoin derivatives of the carrier lysozyme was as described elsewhere^{22,25}. Repetitive yields of the protein carrier and of the radioactively labelled materials ranged between 94 and 96%. Absolute yields corrected for background and carry over²⁶ of the radioactive amino acids studied were 90.6% (*a*), 84.7% (*b*), 81.6% (*c*) and 72.1% (*d*, ○) for chemically deformylated products and 53.0% without chemical deformylation (*d*, ●).

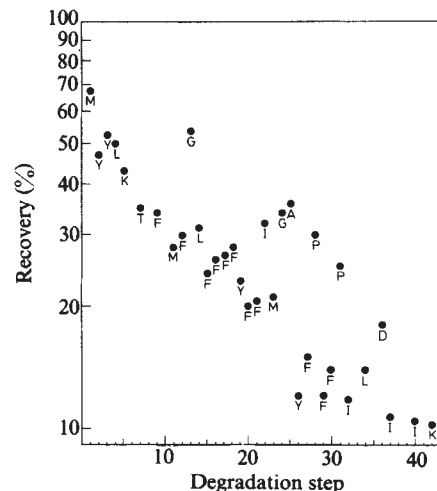


Fig. 4 Partial amino-terminal sequence (residues 1–42) of *lac* permease synthesised *in vitro*. Cell-free products programmed by pGM21 plasmid DNA were labelled with ^{35}S -methionine and the following ^{14}C -labelled amino acids: aspartic acid (D), threonine (T), serine (S), glutamic acid (E), proline (P), glycine (G), alanine (A), valine (V), isoleucine (I), leucine (L), tyrosine (Y), phenylalanine (F), lysine (K) and arginine (R) as described for Fig. 2. The sedimentable material of the protein synthesis mixture obtained by centrifugation at $100,000g$ for 60 min²⁷ was treated and sequenced as described for Fig. 3. The radioactive phenylthiohydantoin derivatives at each sequencer cycle were identified by radiolabelled amino acid analysis after back-hydrolysis with 6M HCl containing 0.2% SnCl_2 (ref. 28) using the Beckman 121M amino acid analyser with effluent collection in principle as described by McKean *et al.*²⁹. The efficiency of radioactive counting was 33% for regenerated ^{14}C -labelled amino acids. The recoveries based on specific activity measurements are plotted on a logarithmic scale against step number²⁶. The relative specific activities of the amino acids were estimated by radiolabelled amino acid analysis of particulate matter after acid hydrolysis and are based on the amino acid composition of *lac* permease deduced from the DNA sequence¹². No correction for hydrolytic loss of the regenerated amino acids was made. Total radioactivity loaded on the sequencer was 2×10^6 c.p.m.

	1	5	10	15
<i>a</i>	Protein	Met-Tyr-Tyr-Leu-Lys- X -Thr- X -Phe- X -Met-Phe-Gly-Leu-Phe-		
	<u>in vitro</u>			
<i>b</i>	Protein	Met-Tyr-Tyr-Leu-Lys-Asn-Thr-Asn-Phe- X -Met-Phe-Gly-Leu-Phe-		
	<u>in vivo</u>	—————		
<i>c</i>	DNA	Met-Tyr-Tyr-Leu-Lys-Asn-Thr-Asn-Phe-Trp-Met-Phe-Gly-Leu-Phe-		
	sequence			

Fig. 5 Summary of sequence data on *lac* permease. *a*, Partial amino-terminal sequence analysis of the *in vitro* synthesised permease (see Figs 3, 4); *b*, automated (underlined residues) and manual sequence analysis of permease isolated from cytoplasmic membranes; *c*, from DNA sequence (12). X, not determined.

vivo product could be determined by the 4-*N,N*-dimethyl-aminoazobenzene 4'-isothiocyanate/phenylisothiocyanate double-coupling method of Chang *et al.*¹⁷. Automated Edman degradation was carried out for five cycles to determine the amount of the amino-terminal residue which could be released by sequential degradation. Methionine was found in the first sequencing step with a yield of 65%. Figure 5 compares the amino-terminal sequences of permease synthesised *in vitro* and *in vivo* with that deduced from the DNA sequence of the *Y* gene. The agreement of the N-terminal sequences confirms the identity of these two products. The amino acid composition determined for the *in vivo* product is very similar to that deduced from the DNA sequence (Table 1). There is good agreement of the values for Glx, Pro, Gly, Val and Arg, each of which occur twice in the C-terminal 15 residues of the permease as deduced from the DNA sequence¹². Any C-terminal processing would then seem unlikely also. The value for phenylalanine is, however, too low. Even after 192 h of hydrolysis, only 35 of the expected 52 phenylalanine residues are found. The unusually high number of potentially acid-resistant Phe-Phe linkages in the sequence¹² may account for the low recovery of phenylalanine from the acid hydrolysate¹⁸ of the *lac* permease.

Conclusions

The N-terminal sequence of both the *in vitro* and *in vivo* products of the *lac Y* gene unambiguously assigns the translational start of the protein to the methionine codon corresponding to base pairs 106–108 in the sequence of Büchel *et al.*¹² rather than to the alternative codons at base pairs 136–138 or 172–174. The identity of the *in vivo* and *in vitro* synthesised proteins in terms of apparent MW and N-terminal sequence shows that this protein is not processed by proteolytic cleavage at the N-terminus. Moreover, both products are deformylated at

the N-terminal methionine, suggesting that the amino-terminal residue is not directly inserted into the membrane and thus shielded against the action of the deformylating enzyme. Other membrane-associated *in vivo* products of bacterial and mitochondrial origin have been shown to retain the fMet residue⁶. There is good agreement between the amino acid composition of the *in vivo* product and that predicted from the DNA sequence. Furthermore, preliminary experiments show that cleavage of the *in vivo* product by cyanogen bromide yields the expected number of polypeptide fragments. These observations make it unlikely that processing at the C-terminus occurs. The MW predicted from the DNA sequence (46,500 (ref. 12)) is much larger than the apparent MW of permease on acrylamide gels. This behaviour could be attributed to increased binding of SDS, as expected from the high hydrophobicity of this protein.

As the cell-free extract used in our studies has not been freed of membranes, it is not clear whether the protein becomes associated with membranes during or after synthesis. Preliminary experiments indicate that the *in vitro* product is readily pelleted by centrifugation but remains more susceptible to proteolysis by trypsin than the functional protein in the membrane, suggesting that the major part of the *in vitro* formed protein is in a non-membranous environment. Whether the lactose permease is synthesised *in vivo* on membrane-bound ribosomes is particularly relevant in view of the location of the *Y* gene between two genes which are probably translated by free ribosomes. Alternatively, the initial formation of a soluble (possibly modified) form of the protein which spontaneously inserts into the membrane cannot be excluded. Obviously, the highly hydrophobic N-terminus of the permease may serve as an initial recognition site for integration into the membrane.

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Sequence of the lactose permease gene

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The nucleotide sequence of the lacY gene coding for lactose permease (M protein) in Escherichia coli has been determined. The sequence includes the intergenic regions between the lacZ (β -galactosidase) and lacY genes as well as the region between the lacY and lacA (transacetylase) genes. Lactose permease is predicted to consist of 417 residues (71% nonpolar), resulting in a protein with a molecular weight of 46,504. The reading frame was confirmed by the sequence of a nonsense mutation changing codon 33 from UGG to UAG.

LACTOSE PERMEASE is a protein which transports and accumulates galactosides through the inner membrane into *Escherichia coli*. It was discovered by Rickenberg *et al.*¹, who described it as the product of a structural gene *Y* of the *lac* system of *E. coli* capable of transport and intracellular accumulation of exogenous galactosides. The product of the *lacY* gene (M protein) was later identified and partially purified in inactive form by Fox and Kennedy². Recently, a plasmid has been described which overproduces *lac* permease³. However, the isolation of inactive *lac* permease has been difficult and the isolation of active permease has not been achieved.

lac permease probably operates without the help of any other protein. We considered that an understanding of its structure would lead to the understanding of its function, transport, therefore we have determined the nucleotide sequence of the *lacY* gene. The sequence allowed the prediction of the primary structure of *lac* permease: the primary translation product consists of 417 residues. Ehring *et al.* (see accompanying article⁴) have shown by protein sequence analysis that *lac* permease synthesised *in vivo* and *in vitro* starts with the native sequence predicted by us.

Cloning of the lacY gene

We used the transducing phage λ h80dlaccI857Ssus7 (ref. 5), carrying the wild-type *lac* operon, as the source of *lacY* DNA. A DNA fragment containing the *lacY* gene was obtained by restriction endonuclease digestion with *EcoRI* and cloned in the filamentous single-stranded DNA phage M13mp2 (refs 6, 7) (for details see Fig. 1 legend). We expected the insert to be 18,000 base pairs long. The DNA length of the cloning vehicle M13mp2 is about 7,200 base pairs, and so an insert of 18,000 base pairs would increase its length about 3.5-fold. Phages of that size would replicate rather slowly, favouring the accumulation of phages carrying deletions in the population. Indeed, we found inserts to have a size of about 5,500 base pairs. One of them was again reduced *in vivo* to 2,100 base pairs (M13mp2561). This insert contained the last 17 codons of the *lacZ* gene, the *lacY* gene and part of the *lacA* gene. We also cloned it in the inverted orientation in M13mp2 and in the mini ColE1 plasmid pVH51 (ref. 8). The latter plasmid (pGB2601) served as a source of smaller restriction subfragments which were required for sequence analysis.

Expression of the *lacY* gene under control of the *lac* promoter of the phage M13mp2 was shown on minimal agar plates containing 0.2% melibiose (see Fig. 1a legend). Although we had expected bacteria carrying about 200 copies of the replicative form (RF) of M13mp2561 DNA to overproduce *lac* permease, in such bacteria, we found that the transport activity was only 50% of that measured in a strain carrying one copy of the *Y* gene on a F'-*lac pro* episome. Therefore, it was possible that we had selected one or several mutations which reduced either the amount or the activity of permease. It was thus essential to compare the DNA sequence of the *lacY* gene cloned in conditions preventing expression with the sequence of the *Y* gene cloned under selective pressure for expression. We

inserted a 3,500-base pair *EcoRI*-*BglII* fragment of λ h80dlaccI857Ssus7 in M13mp2 DNA (Fig. 1b). This fragment carried the *lacY* and the *lacA* genes. The transducing phages thus obtained (M13mp2702) were grown in a *cap cya* strain which reduces *lac* expression to about 2% (ref. 9). The inverted orientation of the fragment was derived by inserting it into M13mp3 DNA⁷ (M13mp3110).

To confirm the reading frame of the *lacY* gene and to define the start of permease, the *Y* amber mutation NE4, mapping in the fourth deletion group of the *Y* gene¹⁰, was directly crossed into M13mp2561 from an F'-*lac pro* episome (see Fig. 1 legend).

DNA sequence analysis

We determined the nucleotide sequence of the *lacY* gene using the methods described by Maxam and Gilbert¹¹ and by Sanger *et al.*¹². For the chemical modification technique¹¹ we prepared restriction subfragments of the 2,100-base pair insert of pGB2601. We constructed a restriction map of *MspI*, *HhaI*, *HaeIII* and *MboII* restriction endonuclease cuts (Fig. 2). Other restriction cuts were deduced during sequence analysis. Single-stranded DNA of M13mp2561, mp2601, mp2702 and mp3110 was prepared¹³ from the respective phages and used as template for sequence analysis according to the method of Sanger *et al.*¹² modified as described by Schreier and Cortese¹³. Primers were prepared as restriction subfragments from the 2,100-base pair insert of pGB2601. Details of the sequencing procedures are given in Fig. 2 legend.

The *lacY* gene sequence of the 2,100-base pair insert, including the intergenic region between *lacY* and *lacA*, was determined on both complementary strands using primarily the enzymatic sequencing method¹². The sequence of the end of the *lacZ* gene and the intergenic region between *lacZ* and *lacY* was obtained from one strand by both the chemical¹¹ and the enzymatic¹² procedures, and the beginning of the *lacA* gene from one strand was obtained by the technique of Sanger *et al.*¹². The *Y* gene sequence of the 3,500-base pair fragment and that of the amber mutant NE4 were determined from one strand by the method of Sanger *et al.*¹². A comprehensive description of the sequencing experiments is given in Fig. 2 legend.

Nucleotide sequence

The DNA sequence (Fig. 3) beginning with the *EcoRI* cut at the end of the *lacZ* gene shows the 17 C-terminal codons of *lacZ*. The sequence is compatible with the protein sequence determined by Fowler and Zabin¹⁴. The end of the *Z* gene is characterised by an unusually strong stop signal: three ochre codons in a row.

In the DNA following the *Z* gene only one frame is free of barriers. To confirm the reading frame of the *lacY* gene we sequenced DNA carrying the amber mutation NE4 mapping in a region of the gene specifying the N-terminus of permease¹⁰. Gels with the respective sequences of NE4 and wild-type DNA are shown in Fig. 4. The guanosine at position 203 is changed to an adenosine, changing a Trp codon (TGG) of the permease

Fig. 1 Cloning of the *lacY* gene. *a*, *EcoRI* restriction endonuclease fragments of the *lac* transducing phage λ h80dlacc1857Ssus7 DNA were ligated with M13mp2 RF DNA linearised by *EcoRI* using T₄ DNA ligase according to Bolivar *et al.*²⁵ The ligation mixture was used to transform strain 75X [*lac*, *pro*]Δ*thi*, *strA*, ϕ 80dlac [*Y*, *A*, *tonB*, *trp*]Δ75X (ref. 10) as described by Mandel and Higa²⁶. Bacteria were selected for growth at 42 °C in supplemented minimal medium²⁷ containing 1% melibiose as carbon source. Cells expressing lactose permease activity will grow in these conditions and enrich in the culture. Due to the F⁻ phenotype of strain, 75X M13 phages can be extruded from the cells but do not re-infect uninfected bacteria. This prevents an excessive propagation of non-hybrid phage as well as the enrichment of the progeny originating from only a few transducing phage by re-infection. Phage from the supernatant of enriched cultures were used to infect strain MUA2 [*lac*, *pro*]Δ*thi*nalA, F' *lac*^IY⁻ΔMUA2 *pro*⁺ (ref. 10) and plated on minimal melibiose plates to grow at 42 °C. Single colonies were assayed for the release of *lacY* transducing M13 phages in the following way. The supernatants of 1-ml cultures were spotted onto a lawn of indicator strain 50-15 [*lac*, *pro*]Δ*thi*, *strA*, F' *lac*^IY⁻ΔMUA2 *pro*⁺ on minimal melibiose plates containing 0.02% streptomycin to select against the former host. The plates were incubated for about 18 h at 33 °C and then at 42 °C for an additional overnight growth period. In these conditions M13mp2 phage give rise to slightly turbid 'plaques' whereas *lacY* transducing phage M13 cause accelerated growth of the infected cells, resulting in a very turbid plaque with a significant halo of bacterial growth at its borderline. In this kind of plaque test, M13mp2 *lacY* transducing phage can easily be distinguished. In addition, suppression of *lacY* amber mutants and recombination to *lacY*⁺ can be monitored directly by the plaque morphology as described below. Two *lacY* transducing M13 phages from different enrichment cultures were obtained. The length of the inserted DNA was determined to be 5,500 base pairs according to its mobility on 1% agarose gels. As the insert, although already shortened by a large deletion, contained four times more DNA than the approximate length of the *lacY* gene, an additional deletion was selected *in vivo*. This final *lacY* transducing M13mp2 derivative contained an insert of 2,100 base pairs (M13mp2561). For the sequence analysis according to Sanger *et al.*¹², a M13 *lacY* derivative with the insert in the inverted orientation was constructed by cutting M13mp2561 DNA with *EcoRI* and then ligating. After transformation, cells were assayed for plaque-forming centres in the *lacY* transduction test described above. Phages carrying the *Y* gene insert in the inverted orientation gave rise to a mottled plaque morphology due to occasional recombination of the Y⁻ host to wild type during plaque development (M13mp2601). A derivative of M13mp2561 carrying the Y⁻ amber mutation NE4 (ref. 10) was derived by propagating the phage in a strain that carried the mutation on a F' *lac pro* episome. *lacY*-negative M13 transducing phages were identified in the plaque test described above. The suppressible character of the mutation was shown by its intermediate phenotype between Y⁻ and Y⁺ using a *supF* host. The correct map position was confirmed by deletion mapping against a set of *lacY* deletions¹⁰ and the failure to recombine with the original NE4 mutation. *b*, To obtain clones in non-expressing conditions a 3,500-base pair *BglII*-*EcoRI* fragment was prepared from plasmid pBR322-Y46, a derivative of pBR322 (ref. 25) containing a 12,000-base pair *EcoRI*-*Bam*HI insert of λ h80dlacc1857Ssus7 including the *lacY* gene. M13mp2 RF DNA was linearised with *EcoRI*, partially digested with *Sau*3A and ligated to the 3,500-base pair *EcoRI*-*BglII* fragment. The *cap*⁻ *cya*⁻ strain CA7914-3 [*lac*, *pro*]Δ*cap*⁻ *cya*⁻ *thi* *strA* F' *lac*^IY⁻ *pro*⁺ was transformed. In induced conditions this strain allows only 2% expression of the *lac* operon⁹, in repressed conditions in the I^Q (*lac* repressor overproducing) situation, even less. Hybrid phages were assayed by the plaque morphology test described above; *lacY* transducing M13 phage showed the typical very turbid plaque (M13mp2702). To obtain a phage with the *EcoRI*-*BglII* insert an inverted orientation, the fragment was inserted into M13mp3 (ref. 7) in the same way as described above. *lacY*-transducing phage of this type give rise to mottled plaques in the described conditions (M13mp3110).

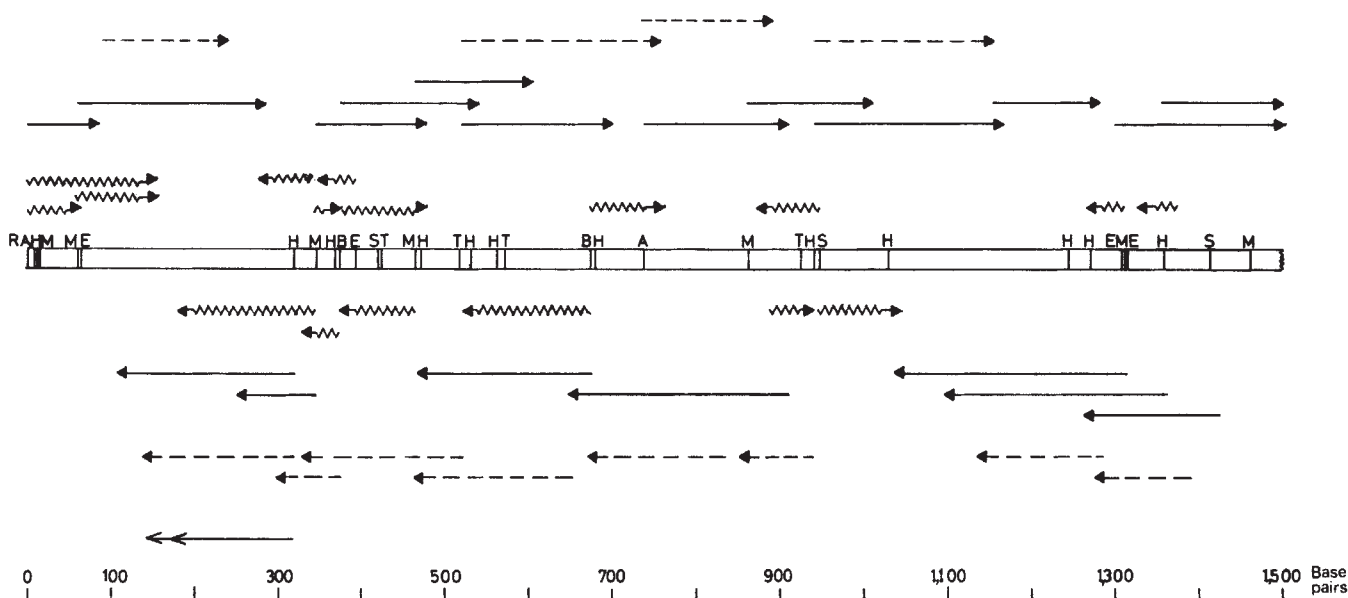
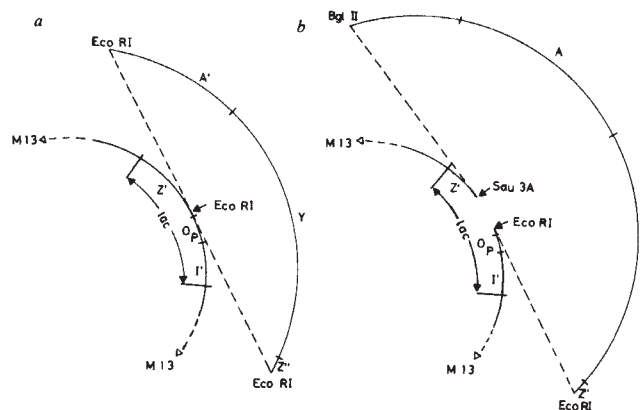
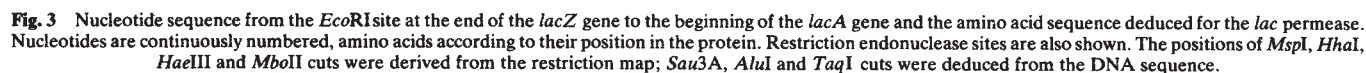


Fig. 2 Restriction map of the *lacY* gene. The sequenced part of the 2,100- and the 3,500-base pair fragments is shown. A (*AluI*), B (*BglII*), E (*EcoRI*), H (*HhaI*), M (*MspI*), R (*EcoRI*), S (*Sau3A*) and T (*TaqI*) denote the respective restriction cuts. For the Maxam-Gilbert sequencing technique¹¹ restriction fragments were labelled at the 5'-end with [γ ³²P]ATP (NEN) by polynucleotide kinase or at the 3'-end by filling in with the suitable [α ³²P]dNTP (NEN) using the DNA polymerase I Klenow fragment. For the sequencing method with chain-terminating inhibitors^{12,13}, templates were prepared according to Schreier and Cortese¹³, restriction subfragments of the *lacY* insert of pGB2601 were used as primers. The same restriction fragments were hybridised to the templates carrying the inserts in opposite orientation so as to obtain the sequence of both complementary strands. Primers of more than 80 base pairs were reduced in length by treatment with exonuclease III before hybridisation with the template (R. Roberts quoted in ref. 13). This yields sequence information on about half of the length of the primer itself. The beginning, direction and extent of the sequence analysis of different parts are indicated by arrows. Arrows above or below the restriction map represent the sequences of the complementary strands determined (5'-end on left-hand side above the map). Wavy arrows represent sequences of the 2,100-base pair insert obtained according to Maxam and Gilbert, and straight arrows those obtained according to Sanger *et al.* Dashed arrows indicate sequences determined from M13mp2701 and M13mp3110 by the technique of Sanger *et al.*; the double arrow represents the sequence determined from the phage carrying the *lacY* amber mutation NE4 (M13mp2561-NE4) according to Sanger *et al.*



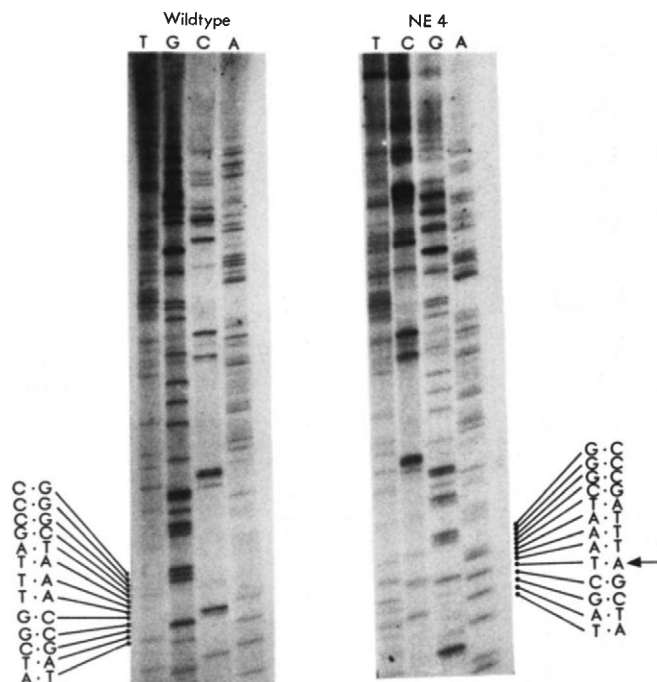


Fig. 4 Autoradiographs of sequencing gels according to Sanger *et al.*¹². The sequence of the region carrying the NE4 mutation in which the Trp codon TGG no. 33 of the *lac* permease gene is changed into the amber triplet TAG is compared with the wild-type sequence of the same region.

gene into the amber triplet TAG. The sequence analysis of this mutation confirmed the suspected reading frame of the *lacY* gene and limited the possible start points. There are only three ATG and no GTG triplets preceding the NE4 amber codon. Only one of the ATG codons is preceded in a proper distance by a 'Shine-Dalgarno sequence'. Thus, we suspect that the *lacY* gene starts after an intergenic region of 45 nucleotides with an ATG codon and ends 1,251 base pairs later with a single ochre codon. The start codon is preceded at a distance of 8 base pairs by the sequence TAAGGA, which can hybridise to the 3'-end of the 16S ribosomal RNA HO-AUCCU determined by Shine and Dalgarno¹⁵. The *lacY* gene recognition site for the 16S rRNA is two nucleotides longer than that of the *lacZ* gene, which is AGGA¹⁶. The sequences of the end of the *lacZ* gene and the beginning of the *lacY* gene have a high A and T content and show a palindromic symmetry. Figure 5a shows a possible secondary structure of the mRNA forming a stem from positions 47–87 and 155–118. The loop of 30 bases contains the 16S ribosomal RNA recognition site and the AUG start codon of the *lacY* gene. Similar secondary structures were proposed by Selker and Yanofsky¹⁷ for *trpB*, *trpE*, *trpG* and *galT* as well as for *lacI*.

The nucleotide sequence from base pair 1,426 to 1,500 (Fig. 3) is compatible with the first 25 amino acids of transacetylase determined by Zabin and Fowler¹⁸. The start codon for fMet-tRNA cannot be unambiguously deduced from the DNA sequence. An interesting possibility is the TTG codon preceding the AAC asparagine codon. TTG has been reported only as a start codon of a restart fragment of the *lac* repressor^{19,20}. A less likely possibility is the GTG codon which lies four triplets upstream of the AAC coding for the N-terminal asparagine. No possible secondary structure of the mRNA could be seen. A ribosomal binding site precedes the UUG in a distance of three bases. The sequence UAACGGAGUGAUC is able to form—with interruptions—of several base pairs with the 3'-end of the 16S ribosomal RNA (Fig. 5b).

The sequence of the *lacY* gene as well as the preceding ribosomal binding site carried by the 3,500-base pair *EcoRI*-*BglII* fragment cloned without any selection for expression

proved to be identical to that of the 2,100-base pair fragment. Thus, we can exclude the possibility that we isolated a *lacY* mutation in our first isolation procedure. We have also cloned the 2,100-base pair fragment in the multicopy plasmid pUR2 (ref. 21). On this plasmid the *lacY* gene is expressed under the control of the *lac* promoter. Strains containing this hybrid plasmid showed at least a fivefold increase in permease activity compared with a strain with the *Y* gene on the *F'*-*lac pro* episome (P. Betteridge, personal communication). Thus, competition of permease and M13 coat protein mRNA for a limiting factor or competition of the proteins themselves for space in the bacterial membrane are possible explanations for the low permease activity in strains harbouring the phage M13mp2561.

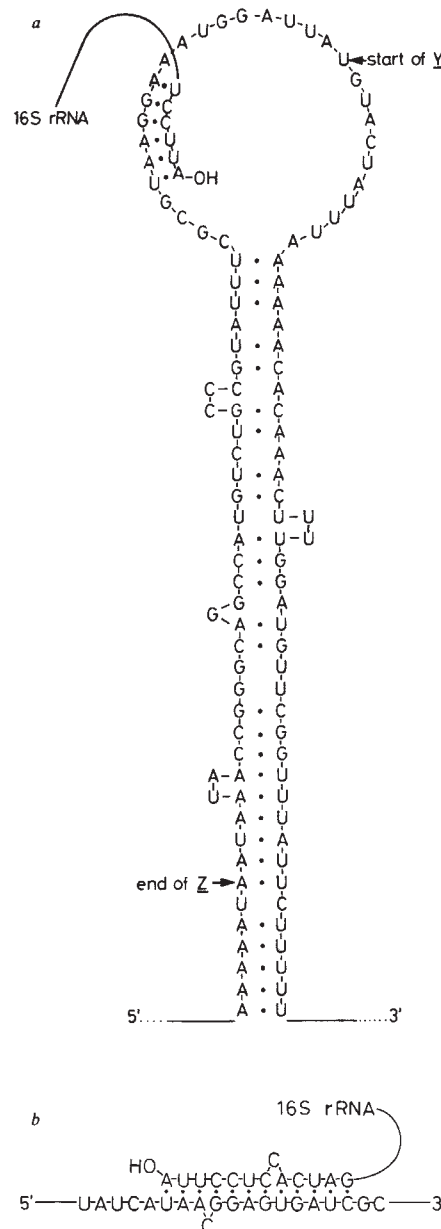


Fig. 5 Ribosomal binding sites of the *lacY* and *lacA* genes. *a*, A possible secondary structure of the mRNA at the beginning of the *lacY* gene spanning the region from nucleotide positions 46 to 155 and exposing the ribosomal binding site and the AUG start codon in the loop. *b*, Possible extent of hybridisation of the 3'-end of the 16S ribosomal RNA to the mRNA at the beginning of the *lacA* gene.

Protein sequence of *lac* permease deduced from the DNA sequence

Ehring *et al.* (see accompanying article)⁴ found that *lac* permease synthesised *in vivo* and *in vitro* started with the N-terminal amino acids predicted for native permease from the DNA sequence. The DNA sequence suggests that it consists of 417 amino acids (Fig. 3), resulting in a molecular weight of 46,504. This is higher than the 29,000 reported by Jones and Kennedy²² and the 30,000 reported by Teather *et al.*³. A similar size was found by Ehring *et al.*⁴ for permease synthesised *in vivo* and *in vitro*. Thus, either the membrane protein *lac* permease behaves atypically on SDS gels or it is proteolytically degraded at its C-terminus.

lac permease is a strongly basic protein of low polarity (29% polar, 71% unpolar); the high content of phenylalanine residues, 56 out of 417, is remarkable. Of these, 22 are clustered in the N-terminal region, making it highly hydrophobic. The first N-terminal lipophilic part of the protein spans 26 amino acids at positions 9–34 and has a hydrophobicity index of 4.0 determined according to Segrest and Feldmann²³. It is followed in short distances by three additional hydrophobic sequences at positions 45–66, 76–99 and 108–118, with hydrophobicity values of 2.5, 3.4 and 2.4, respectively. The middle part has a more hydrophilic character, containing charged and less hydrophobic amino acids. This region, from residue 119 to 274, is

separated into two parts by a lipophilic sequence of 11 amino acids (177–187), with a hydrophobicity index of 3.3. The C-terminus again contains hydrophobic sequences in clusters: residues 275–283, 291–299, 326–334, 347–357 and 381–395, with corresponding hydrophobicity indices of 3.7, 2.3, 2.7, 3.0 and 2.7.

We conclude that we can divide lactose permease into four structural parts: a hydrophobic N-terminus, two hydrophilic regions in the middle part of the protein, separated by a lipophilic sequence of 11 amino acids, and a hydrophobic C-terminus. We propose that the hydrophobic regions at the N-terminus and the C-terminus function as anchors by which the protein is fixed in the membrane; they could also form pores for galactoside and proton transport. The length of the lipophilic sequence from residue 177 to 187 separating the two hydrophilic parts may be sufficient to penetrate the membrane. Thus, the function of sugar recognition and binding may be carried out by the two hydrophilic domains which could occupy sites inside and outside the membrane. Genetic and biochemical data support the idea that *lac* permease is active as an oligomer, possibly as a dimer²⁴.

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Structure of the junction between communicating cells

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An 18-   resolution map of the 'gap junction' has been obtained by electron microscopy. The protein oligomer in the junctional membranes, the 'connexon', is a cylinder composed of six subunits which are tilted around its axis. Analysis of two different subunit configurations suggests how the connexon might regulate the passage of small molecules between cell interiors.

SPECIALISED regions of contact known as gap junctions¹ exist between apposed plasma membranes of communicating cells. These junctions are believed to mediate and regulate the passage of ions and small molecules between the cell interiors². They are composed of units, called connexons³, which are embedded in the apposed membranes, in register, and linked to each other. The connexon is often organised on a regular hexagonal lattice, as first described in detail by Robertson⁴. Its structure in this lattice has been studied by electron microscopy^{5,6} and low angle X-ray diffraction^{7,8}. It is thought to be constructed from protein oligomers arranged in the form of an

annulus and delineating a narrow channel through the membrane. This channel is presumed to provide the regulated hydrophilic pathway connecting the cytoplasm of adjacent cells.

Experiments with fluorescent tracer molecules (see, for example, refs 9, 10) suggest that the channel can accommodate a wide range of molecular sizes (up to about 800 daltons in mammalian cells¹⁰). Cytoplasmic free Ca²⁺ concentration¹¹ and/or pH (ref. 12) may be involved in regulating its permeability. Although electron microscopic and X-ray evidence point to changes in the size¹³, shape and packing of the connexon^{8,13}, it is

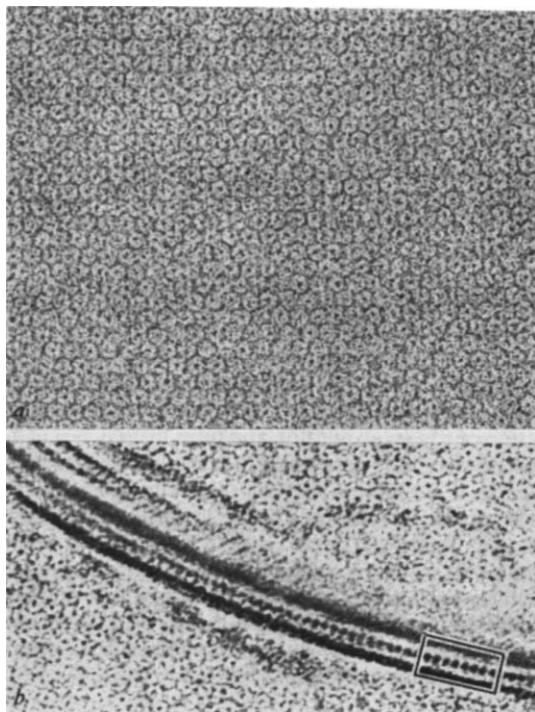


Fig. 1 Electron micrographs of isolated gap junction sheets viewed from a direction perpendicular to the plane of the membranes (*a*) and a direction more nearly parallel to this plane (*b*). In *a* the connexons are seen as annular units organised on a hexagonal lattice, with unit cell dimension 85 Å; in *b* they are seen as bridges connecting the two apposed membranes. The view in *b* arises from a fortuitous curling up of a sheet at its edge; the region outlined (looking along the [1, 1] direction) was used to estimate the average density profile in the direction perpendicular to the plane of the junction, and hence the variation in amplitude and phase along the lattice line through the origin. Uranyl acetate negative stain. $\times 300,000$.

not clear how changes in the structure cause the permeability changes.

We have studied the structure of isolated gap junctions to a resolution of 18 Å by electron microscopy of specimens tilted at various angles to the incident beam. The three-dimensional maps reveal the configuration of the connexon subunits, their relationship to the membrane surfaces and the nature of the region they enclose, within the membrane. The subunits are found to take up two alternative configurations; the differences between them suggest a simple molecular mechanism by which the permeability may be controlled *in vivo*.

Electron microscopy

We used rat hepatocyte junctions, isolated and negatively stained as described previously¹⁴. *En face* views show the (superimposed) connexons as annuli arranged on a hexagonal lattice of unit cell dimension 85 Å (Fig. 1*a*). Edge-on views show the connexons as links which bridge the extracellular space between the apposed membranes, creating a gap of 30–40 Å (Fig. 1*b*). These junctions seem to be the same as those isolated by others (for example, ref. 15). They can be converted to a second form having the same hexagonal lattice, but in which individual connexons are less distinct, by dialysing them against water at 4° C for several days¹⁴. We shall describe below how the normal form, as in Fig. 1, and this second form differ in their three-dimensional structure.

We investigated the structure of the normal junctions by Fourier methods^{14,16,17}. Specimens were tilted at angles of

between 0° and 79° to the incident 100-kV electron beam, and micrographs were recorded using low electron doses to minimise the effects of radiation damage. Densitometry and computer processing were carried out on selected micrographs¹⁴ from several specimens to give the Fourier terms into which the structure can be analysed. The three-dimensional Fourier transform consists of a set of reciprocal lattice lines, perpendicular to the plane of the junction, along which the amplitudes and phases vary continuously. The Fourier terms determined from micrographs taken with different tilts give the values of the amplitudes and phases at different sections through these lattice lines. To map out the continuous variation along each line, the terms were combined, in the first instance, according to the lowest symmetry compatible with the hexagonal projected structure, that of the crystallographic space group, P3. We began with data from specimens recorded with smallest tilt, and only compared values which along any given line lay within 0.0025 Å⁻¹ of each other. Twenty-six micrographs were used, each providing up to 30 independent measurements of amplitude and phase. The average phase error, based on comparisons between each new measurement and all previously accumulated phases related by symmetry, was 21°. Smooth curves, then drawn through the combined data, demonstrated that points equidistant from the midpoint along each lattice line had equivalent amplitudes and opposite phases. This result indicated that, at least to the resolution of the analysis, the junction had the higher symmetry of the space group, P6. Therefore, to improve the accuracy, the data were refined a second time according to this latter space group.

The amplitude and phase curves (Fig. 2) show minor departures from the symmetry of the space group, P6₂₂, relating the two oppositely facing halves of the junction by dyad axes lying in its central plane. Therefore, the two halves are almost, but not exactly, equivalent. The departures from perfect symmetry were probably produced as the stain dried around the specimen and support film. In favour of this argument, we found that the phase error introduced in combining data from different junctions depended on whether we took the surface next to the support film to be at the top or bottom of the structure. In all cases, the assumption of a consistent location for the support film gave the smallest value for this error.

It was useful, although not critical for an understanding of the structure, to estimate the average density profile in the direction perpendicular to the plane of the junction, and hence the variation in amplitude and phase along the lattice line through the origin (the 0,0 lattice line). Edge-on views of the junction, as seen in Fig. 1*b*, were analysed for this purpose. Fourier transforms calculated from these views gave directly the variations along the 0,0 and 1,1 lattice lines. Correlation with the 1,1 lattice line derived from the combined data then allowed us to select the most accurate 0,0 lattice line and determine the scale for the variation along it. Its peak amplitude was found to be six times that of the 1,1. We assumed that the profile corresponding to this variation was reasonably correct because it could be closely matched to features in a preliminary three-dimensional map obtained without the 0,0 lattice line included in the calculations.

The complete transform consisted of 11 crystallographically independent lattice lines (0,0; 0,1; 0,2; 0,3; 0,4; 1,1; 1,2; 1,3; 2,1; 2,2; 3,1), and extended in all directions to a resolution of about 18 Å. We collected 192 Fourier terms by sampling the smooth curves at intervals of 0.0025 Å⁻¹ along these lines.

Three-dimensional map

The map calculated by Fourier synthesis of these terms depicts the membrane surfaces and the hydrophilic surfaces of the connexon subunits, as these are the parts of the structure exposed to the negative stain. In Fig. 3 we consider one half of this map, corresponding to one of the two oppositely facing halves of the junction. There is a rather featureless zone, presumed to be the membrane, with matter on either side

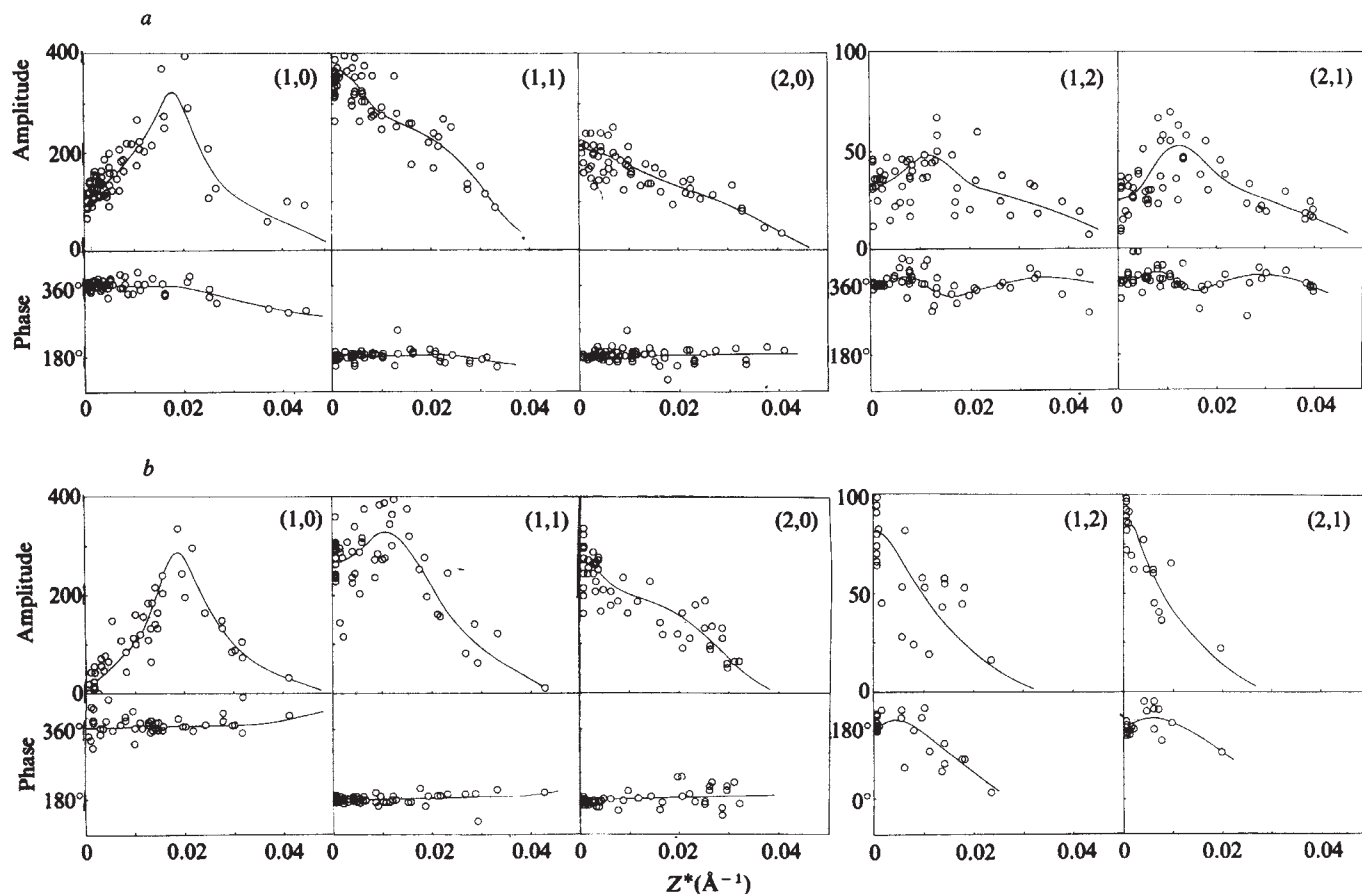


Fig. 2 Values of amplitude and phase determined along reciprocal lattice lines from micrographs of specimens tilted to the incident electron beam by angles up to 79° ; *a*, the normal junction (as in Fig. 1); *b*, the form after the transition. Z^* is the distance from the midpoint along the lattice line. The tilt range 0 – 55° was obtained conventionally, using a goniometer stage; the additional tilt to 79° was achieved by bending the support grids. The data have been combined assuming the space group, $P6$; thus, the Friedel relationship gives the values for negative Z^* . Smooth curves drawn through the points and sampled at intervals of $0.0025 \text{ \AA}^{-1}$ gave the Fourier terms from which the maps were calculated. The data extend to the points where the amplitudes became comparable with the local background level, that is, to a resolution of $\sim 18 \text{ \AA}$ in *a* and $\sim 23 \text{ \AA}$ in *b*. The high tilt angles ($> 60^\circ$) were required to determine the variation along the $1,0$ lattice line at values of $Z^* \geq 0.02 \text{ \AA}^{-1}$; only the strong low resolution lattice lines ($1,0$; $1,1$; $2,0$) were used in the refinement of these data. The additional lattice lines, obtained for *a*, but not shown, are weak and do not significantly affect the detail in Fig. 4*a*; they improve the definition in Fig. 3, but do not contribute new features.

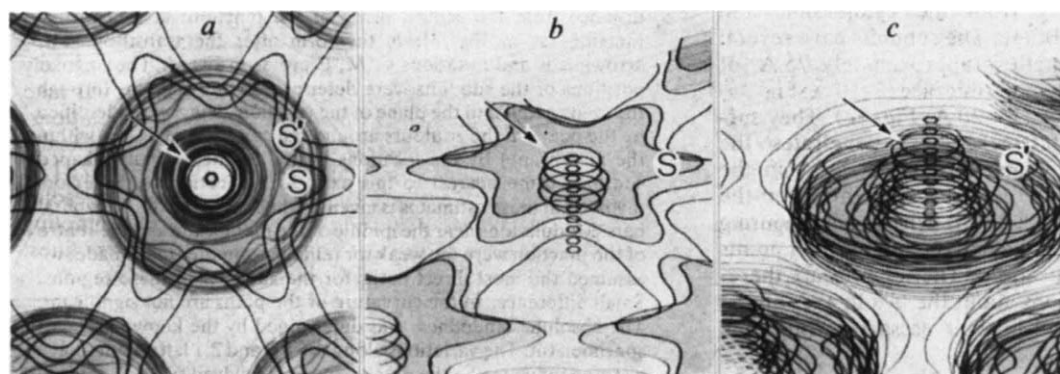


Fig. 3 Map of one half of the gap junction, as seen from the interior of the cell. Sections through the structure were traced onto Perspex sheets, which were then stacked on top of each other; the complete map (*a*) is composed of a portion (*b*) containing the cytoplasmic face, stacked on an equal portion (*c*) containing the extracellular face. The orthogonal view (*a*) emphasises changes in the

angular disposition of the subunits; the oblique views (*b*, *c*) emphasise the subunit features in relation to the membrane surfaces. The contours identify the stain-excluding regions formed by the components of the junction (compare Fig. 4*a*); the exact positions of their boundaries are not accurately determined because these are sensitive to small errors in the variation along the $0,0$ lattice line. The detail protruding from the membrane is emphasised by shading and by additional contours. The arrow points to the opening surrounded by the connexon subunits, one of which is labelled, S and S'. Hexagons have been drawn at the 6-fold axis of the connexon on each of the Perspex sheets. The half of the junction not shown contains essentially the same detail, but is slightly less well contrasted by the stain.

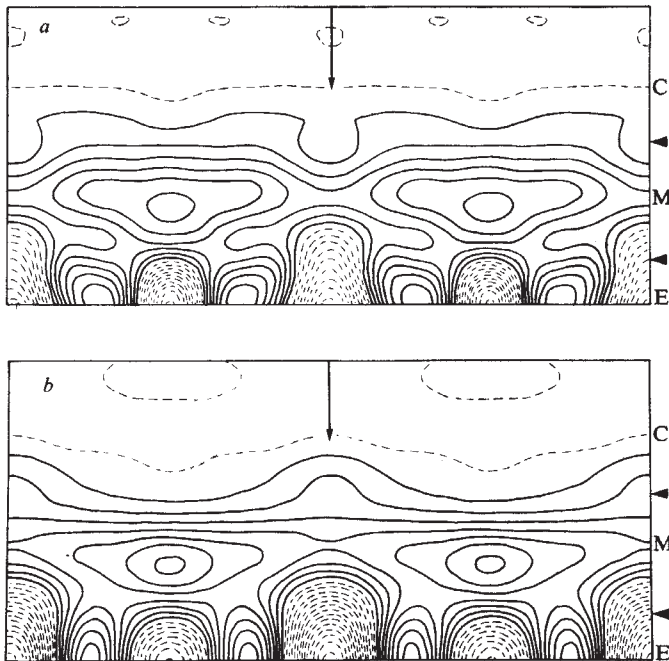


Fig. 4 Sections through the maps perpendicular to the plane of the junction, along a line joining the connexon axes; *a*, the normal junction (as in Fig. 3); *b*, the form after the transition. Arrowheads point to the approximate locations of the cytoplasmic (upper) and extracellular (lower) membrane surfaces. The zones C, M and E refer to the cytoplasm, membrane and extracellular space, respectively. Matter at the cytoplasmic surface moves towards the connexon axis (vertical arrow), closing the opening there, in going from *a* to *b*. This effect is contributed mainly by the change in amplitude along the strong 1,1 lattice line. Contours corresponding to stain-excluding regions (negative contours) are drawn as continuous lines; contours corresponding to stain-filled regions (zero and positive contours) are drawn as broken lines. The same Fourier terms along the 0,0 lattice line were used in the calculations for both maps. The sections contain two unit cells in the horizontal direction and half of the junction in the vertical direction. The absolute scale in the vertical direction is sensitive to flattening effects associated with the stain drying around the specimen²². Assuming a total thickness of 50 Å for the membrane⁸, the connexon must extend about 75 Å from the central plane of the junction.

(shaded in Fig. 3) clustered around the 6-fold axes. The clusters are hexamers, presumed to be the exposed portions of six connexon subunits protruding from the cytoplasmic and extracellular faces of the membrane. The subunits have several features of interest. Their length is approximately 75 Å, of which less protrudes from the cytoplasmic face (5–10 Å; Fig. 3*b*) than from the extracellular face (15–20 Å; Fig. 3*c*). They surround a narrow opening (arrow) which penetrates the membrane and widens to a diameter of about 20 Å in the extracellular region of the junction. They are centred on the cytoplasmic face at points (such as S) where neighbouring connexons come closest, and on the extracellular face at points (such as S') in between these locations. The subunits are therefore at different angular positions about the 6-fold axes on the two faces and must be inclined in their passage through the membrane.

The distinct annular appearance of the connexon in the micrograph (Fig. 1*a*) can now be understood to result from summation of annular features on both the cytoplasmic and extracellular faces. The 6-fold modulations around this annulus are weak, because the modulations at the cytoplasmic and extracellular faces (peaked at S and S') are not in register and to some extent cancel each other out.

Two quaternary states

We investigated the structure of the second form of junction, produced by dialysis. Data were collected and combined from 20 micrographs of specimens tilted through the same range of angles as before; the average phase error, assessed in the same way, was 22°. Figure 2 compares the variations in amplitudes and phases for the two forms. There are large differences along the 1,2 and 2,1 lattice lines where the amplitudes peak at different places and the phases differ by about 180°. There are smaller differences, involving mainly amplitudes, along others. Low angle X-ray diffraction patterns from orientated pellets of isolated gap junctions^{7,8} corroborate the curves obtained for the normal junction (Fig. 2*a*). The first four X-ray lattice lines (0,1; 0,2; 1,1; '1,2') are the strongest, and the 1,2 lattice line is peaked away from the equator. The most pronounced changes that the transition should produce in the X-ray pattern are a convergence of the off-axial peaks on the 1,2 lattice line into a single peak on the equator, and a splitting of the equatorial peak on the 1,1 lattice line into two slightly off-axial ones.

The maps of the junctions in the two states show several differences. These were interpreted in terms of two kinds of displacement of the subunits, one radial and the other tangential about the 6-fold axis through the centre of the connexon. The radial displacement is insignificant near the centre of the junction, but is pronounced near the cytoplasmic face (Fig. 4). The opening delineated there by the subunits (arrow in Fig. 4*a*) disappears after the transition (Fig. 4*b*), indicating movement of matter towards the connexon axis. A radial displacement of the order of 5 Å would be consistent with the maps, but we emphasise that the method of visualising the structure, using

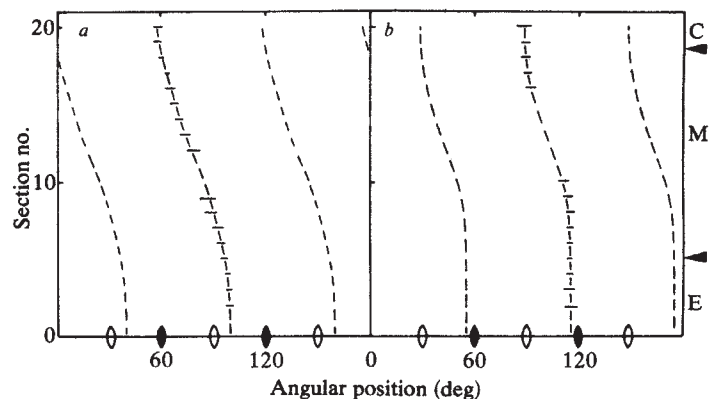


Fig. 5 Two-dimensional net showing the angular positions of the subunits about the 6-fold axis of the connexon in relation to distance from the central plane of the junction; *a*, the normal junction (as in Fig. 3); *b*, the form after the transition. The arrowheads and notations C, M, E are as in Fig. 4. The angular positions of the subunits were determined from sections through the maps parallel to the plane of the junction. They were identified by the peaks in the contours around the connexon axis, or (within the membrane) by the locations where the hexagonally shaped contours came nearest to this axis. An indication of the errors involved in these estimates is given by the lengths of the horizontal bars. Modulations near the middle of the membrane and the centre of the junction were too weak for reliable estimates to be made; we assumed the most direct paths for the subunits in these regions. Small differences in the curvature of the paths are not significant. The absolute handedness was determined by the known sense of specimen tilt. The variations along the 1,2 and 2,1 lattice lines have a strong influence on the details shown. The dyad symbols refer to putative dyad axes lying in the central plane of the junction along the [1,0] (filled symbols) and [1,1] (open symbols) crystallographic directions, and relating the apposed connexons facing each other in the two membranes. Perfect dyads are not present because the two halves of the junction are not quite equally contrasted by the stain. The positions of the subunits in the more poorly stained halves are consistent with the presence of dyads.

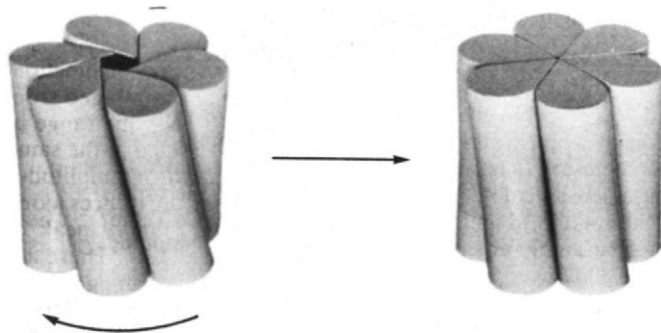


Fig. 6 Wooden model of the connexon, depicting the transition from the 'open' to the 'closed' configuration, as suggested by the maps. It is proposed that the closure on the cytoplasmic face (uppermost) is achieved by the subunits sliding against each other, decreasing their inclination and hence rotating, in a clockwise sense, at the base. The darker shading on the side of the model indicates the portion which would be embedded in the membrane. The subunit inclinations depicted are the average inclinations estimated from the nets (Fig. 5), and the diameter at the base of the connexon, as indicated by our data, is fixed. The radial displacement of each subunit at the cytoplasmic end would be about 6 Å, given the observed inclination change of 5° distributed over its 75-Å length.

negative stain, does not permit an accurate estimate to be made. This redistribution of matter on the cytoplasmic face accounts for the change in contrast of the connexon seen in the micrographs¹⁴. The annular appearance of the connexon would become less distinct after the transition because the detail on the cytoplasmic face no longer superimposes to reinforce the annular detail on the extracellular face.

The tangential displacement about the connexon axis was measured by tracing the paths followed by the subunits from the two maps. Two-dimensional nets were plotted giving the angular positions of the subunits in relation to distance from the central plane of the junction (Fig. 5). The nets show that the subunits follow fairly straight, slightly sigmoidal, paths which are differently inclined in the two structures. Their average inclination is reduced by the transition (made more nearly perpendicular to the plane of the junction) from 14° (Fig. 5a) to 9° (Fig. 5b)—a total change of about 5°. In addition to this tilting of the subunits, the connexon itself rotates by about 23° within the crystal lattice. More subtle effects, for example, distortions of the subunits, may be present but are not resolved. It would be difficult to detect small changes in the lengths of the subunits⁸ by our technique.

The inclination changes produce rotations between equivalent parts of apposed connexons (that is, connexons facing each other in the two membranes) about their common 6-fold axis. The magnitude of these rotations can be estimated from Fig. 5, as the two halves of the junction are related to each other crystallographically (see Fig. 5 legend). The maximum value, 28°, is in the central plane of the junction, and here the subunits of apposed connexons must slide over each other by a distance of about 11 Å.

Structure of the connexon

The features in the three-dimensional map (Fig. 3) indicate that the connexon is an annular oligomer composed of six protein subunits which span the membrane and protrude from either side. Assuming a value of 25–30,000 daltons (refs 14, 18–20) for the molecular weight of the gap junction protein and 1.3 Å³ per dalton for the partial specific volume (the value obtained for another, well studied, membrane protein¹⁷), each subunit must occupy a volume of at least 32,500 Å³. To fill this volume, its cross-section inside the membrane would have to be at least

equal to that outside. We suppose, then, that the subunits are roughly rod-shaped, about 25 Å in diameter and 75 Å long. A picture similar to this was deduced from a combined study by electron microscopy and low angle X-ray diffraction⁸. In addition, our analysis shows a variation in the dimensions of the central opening delineated by the subunits. It is about 20 Å wide in the extracellular region of the junction, but is narrower within the membrane where it is not fully resolved. The path taken by the subunits is also inclined tangentially with respect to the 6-fold axis of the connexon, giving the whole assembly a left-hand character.

The transition to the second configuration of the connexon is produced by a radial inwards motion of the subunits near their cytoplasmic extremities and a reduction of their inclination tangential to the 6-fold axis. These movements are accompanied by a rotation between the subunits of apposed connexons in the central plane of the junction. A comparable rotational displacement, together with tilting of the subunits, occurs between the two layers of the protein disk of tobacco mosaic virus when it undergoes a transition into the helical assembly found in the virus²¹.

We note that radial motion at one end of the connexon could be transmitted through a change of subunit inclination into rotational motion at the other end by a process in which the subunits slide over each other along their lines of contact. The small displacements involved could be accommodated by only very minor distortions of the subunits. Such an action, which is depicted in Fig. 6, seems to describe well the changes in configuration suggested by our maps. The radial displacement of each subunit at the cytoplasmic end should be ~6 Å, given the observed change of inclination of 5°, distributed over its length. Displacement by about this amount is suggested by the details in Fig. 4.

A rearrangement of essentially rigid subunits has not so far been found in oligomeric membrane proteins, but is a common feature of soluble multiple subunit, multi-state assemblies which change their structure and function in response to chemical stimuli. The particular rearrangement in Fig. 6 provides a simple mechanism for opening and closing a narrow channel through a membrane. This mechanism may be the one by which the gap junction regulates the passage of small molecules between living cells. The dimensional changes seem to be of the correct magnitude to account for the observed permeability changes.

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LETTERS

IR flashes from the X-ray rapid burster

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We confirm here the report of Kulkarni *et al.*¹ who discovered IR bursts from the X-ray rapid burster, MXB1730–335. We observed two bursts at 2.2 μm , each of energy $\sim 10^{31}$ J, and a new feature; several bright, brief flashes ($\Delta t < 1$ s) superimposed on the burst continuum. The brevity, luminosity and inferred brightness temperature of the flashes ($T_B \geq 10^{10}$ K) imply a powerful, non-thermal emission mechanism. Simultaneous observations across the whole spectrum with high time resolution would be invaluable in understanding the emission process.

The rapid burster was discovered in 1976 by Lewin *et al.*² at MIT and is unique in producing $\sim 10^3$ X-ray bursts a day in its active periods. These periods occur about every 6 months and last 2–6 weeks³. Liller⁴, and Kleinmann *et al.*⁵ have shown that the source lies in a compact globular cluster, now called Liller 1, at a distance of 10 kpc. By 1978 it had become clear that the same cluster also produces 'special' bursts⁶, similar to those from the more usual X-ray burst sources. They are characterised by a softening of the X-ray spectrum during decay and are now known as Type I bursts. The rapid bursts are classified as Type II. In April 1979 Kulkarni *et al.*¹, observing from Kavalur in India, detected six bursts at 1.6 μm which they attributed to the Type I X-ray bursts.

On 8 August 1979 Type II bursts were seen by the X-ray satellite Hakucho⁷, heralding a new period of activity. We attempted IR observations in early September using the 1.5 m IR flux collector at Izaña, Tenerife. The detector was an indium antimonide diode cooled to 75 K. The cooled filter had an effective wavelength of 2.2 μm and a bandwidth of 0.4 μm . We used a two-mirror focal plane sky chopper⁸ at 20 Hz, with one beam (20 arc s diameter) centred on Liller 1 and the reference beam 20 arc s to the north. For calibration we used ϕ Ophiuchi ($m_K = 2.27$) taking its flux as $F_\lambda = 4.8 \times 10^{-11} \text{ W m}^{-2} \mu\text{m}^{-1}$ in accordance with Johnson's⁹ absolute flux calibration.

Table 1 lists the dates and times of our observing periods; a total of 5 h 15 min. In this time we saw only two bursts, 1 min apart, on 5 September 1979 (Kulkarni *et al.* saw six in 2.5 h). The chart recording of the bursts is shown in Fig. 1. They appeared at 21 h 04 min 01 s and 21 h 04 min 55 s UT ± 1 s, and lasted 20 s

and 10 s respectively. They are similar in shape to the Kavalur bursts, except for the seven sharp spikes accompanying them. Our nominal time resolution, set by the output filter of our phase-sensitive detector (PSD) was 0.3 s, but the widths of two of the spikes (1d and 2c) remain unresolved (see Table 2). The negative-going spike, marked A in Fig. 1, is probably an artefact of the PSD caused by a sudden, rapidly increasing signal. We have been able to reproduce this feature with a laboratory PSD. The other spikes do not seem to be spurious. They appear nowhere else on the chart trace and are closely associated with the structure of the bursts. Assuming they are real, we can properly describe them as flashes.

In Table 2 we list some observed and derived characteristics of the flashes assuming a distance of 10 kpc and an interstellar absorption of 1.1 mag (ref. 5). With the data from Table 2 and Fig. 1 we can make the following observations.

Why are IR bursts visible at all? The MIT group¹⁰ have measured the spectra of several X-ray bursts, both Type I and Type II, and have fitted them to black-body curves of temperature $T \approx 2 \times 10^7$ K. The inferred radius of the X-ray emitting region is $R \approx 10^4$ m. If we extrapolate the black-body spectrum into the IR we would expect a flux $F_\lambda \sim 10^{-23} \text{ W m}^{-2} \mu\text{m}^{-1}$ at 2.2 μm ; a factor of 10^{11} below our observed value.

Two of the flashes are unresolved at our instrumental time resolution of 0.3 s, putting an upper limit on the size of the emitting region of $R = 10^8$ m. This implies an IR brightness temperature $T_B \geq 4 \times 10^{10}$ K. If we assume that the IR radiation is coming from the same small region as the X rays, T_B rises to an improbable 4×10^{18} K.

If the radiation is isotropic, then the IR burst energies in our 0.4- μm bandwidth are 9×10^{30} J and 6×10^{30} J, and the peak luminosity is 2.2×10^{30} W. This compares with $\sim 10^{32}$ J and $\sim 10^{31}$ W for the Type I X-ray bursts³.

It may be significant that the three flashes in each burst occur at similar times after the initial rise (see Table 2), except

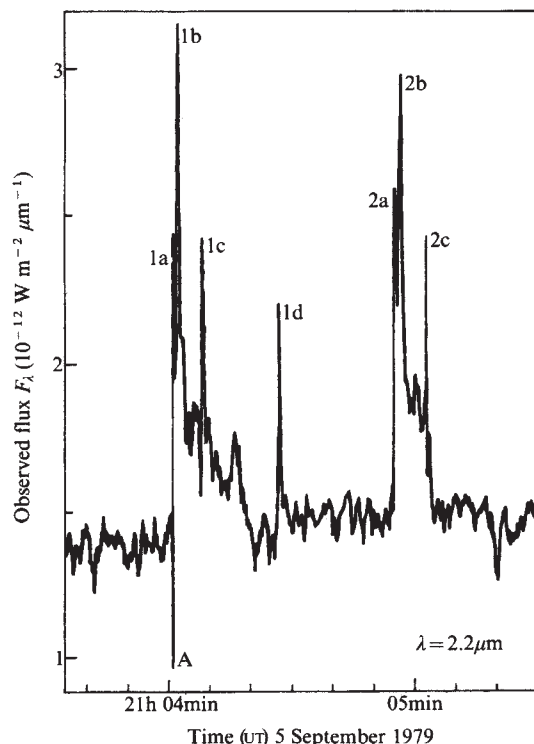


Fig. 1 Two IR bursts from the X-ray rapid burster, accompanied by seven brief flashes. The flux scale is not corrected for interstellar absorption. The feature at A is an artefact of the detection system.

Table 1 IR observations of the rapid burster

Date (September 1979)	Times (UT)				No. of bursts
	Start (h min)	Finish (h min)	Duration (h min)		
5	20 50	22 10	1 20		2
6	20 20	22 05	1 45		0
11	21 10	21 45	0 35		0
12	20 05	21 40	1 35		0
		Total	5 15		2

Table 2 Details of the IR flashes

Flash no.	Time of peak (s ± 0.1)	Half-peak width (s ± 0.1)	In bandwidth Luminosity (10 ³⁰ W)	0.4 μm Energy (10 ³⁰ J)
1a	0.0	0.5	1.3	0.6
1b	1.2	0.9	2.2	1.9
1c	7.0	0.8	1.3	1.0
1d	25.7	0.3	1.0	0.3
2a	54.2	0.6*	1.4	0.8
2b	55.6	1.5	1.9	2.8
2c	61.7	< 0.3	1.2	< 0.4

The half-peak widths, luminosities and energies were measured with respect to the background on either side of each burst. Isotropic radiation is assumed.

*This is the rise time: the half-peak width cannot be measured.

for the solitary flash 1d, which occurs in the space between the bursts.

Although we observed for 5 h 15 min the bursts appeared in the space of 1 min, suggesting that they may be physically associated. Kulkarni *et al.* noted similar groupings among some, but not all, of their bursts.

There are no clear spikes in the Kavalur bursts, but they did have a lower time resolution (0.6 s) and the signals were 2 or 3 times noisier than ours. It is not clear whether flashes would have been detected if they were there. The difference in wavelength could also be important. If the spectral index of the flashes is different from that of the burst continuum, then the flashes may be apparent at one wavelength but not at another.

We do not intend to go into the details of possible mechanisms, but we conclude: (1) As there have been no simultaneous X-ray/IR observations, we cannot say for certain that our bursts correspond to X-ray bursts; Type I X-ray bursts recur at intervals of hours, while the Type II bursts repeat at intervals of seconds or minutes. The IR bursts observed so far do not fit either category.

(2) If our observations do correspond to X-ray bursts, then the high brightness temperature and unexpectedly intense IR flux strongly suggest a non-thermal emission mechanism. The favoured explanation of the Type I X-ray bursts is a thermonuclear helium flash in accreted material on a neutron star^{3,11}. This accounts for the observed X-ray behaviour very well, but does not predict an observable IR flux. Apparao and Chitre¹² propose that the IR bursts are produced by a 'cyclotron maser' operating above the poles of a magnetised neutron star in a close binary system.

Further observations at IR wavelengths should attempt time resolutions of <0.1 s to detect and resolve the flashes. Simultaneous observations at X-ray, optical and several IR wavelengths would be invaluable in understanding the emission process.

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Semi-empirical estimates of gravitational wave generation by stellar collapse

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The problems of the generation and detection of gravitational waves and the difficulty of detecting the burst of gravitational radiation produced by a stellar collapse in our Galaxy, or in neighbouring clusters of galaxies where such collapses are expected to occur frequently, have been reviewed elsewhere^{1–3}. As stellar collapses, explosions, or supernovae are poorly understood, the values of the strength of these sources depend on uncertain assumptions. However, it is possible to make some independent estimates on semi-empirical grounds, using observed facts concerning the remnants of stellar evolution. These estimates, reported here, have serious weaknesses. They must involve oversimplified models and very crude standard calculations of the collapse and radiation processes. Also, because they are based on observed properties of stellar remnants, they say nothing about collapses which do not produce observable remnants. Although this selection may introduce a strong bias, these estimates deserve consideration because they are tied to real data.

Consider a simple model for the collapse of a stellar core of mass m into a dense remnant and expelled debris: ignoring the mass and momentum radiated away, the remnant has a mass $(1 - \alpha)m$ and a velocity αv ; the debris have a total mass αm and their barycentre has a velocity, in the opposite direction to that of the remnant, of $(1 - \alpha)v$. If we assume that the debris cloud acts as a point at its barycentre, and that acceleration of remnant and debris occurs over a time t , then it is easy to estimate the emitted power P , energy E , and efficiency ϵ . The quadrupole moment is given by

$$Q \approx Mv^2 t^2 \alpha (1 - \alpha) \quad (1a)$$

$$|\ddot{Q}| \approx Mv^2 \alpha (1 - \alpha) / t \quad (1b)$$

The duration of the radiation is also about t . Then

$$E \approx Pt \approx \frac{Gt}{5c^5} |\ddot{Q}|^2 \approx \frac{GM^2 v^4}{5c^5 t} \alpha^2 (1 - \alpha)^2 \quad (2a)$$

$$\epsilon \equiv \frac{E}{Mc^2} \approx \frac{GMv^4 \alpha^2 (1 - \alpha)^2}{5c^7 t} \quad (2b)$$

Now we can apply our knowledge of certain classes of collapsed objects. For the X-ray sources in globular clusters a simple argument⁴ based on the dynamical properties of the clusters shows that the recoil velocity of the remnant $\alpha v < 10^{-4}c$. For galactic pulsars $\alpha v \approx 10^{-3}c$. Taking $1 - \alpha \approx 1$ and, with r the size of the exploding core, $t \approx r/v \approx (c/v)(10GM/c^3) \approx 10GM/vc^2$ gives

$$\epsilon \approx \frac{1}{50} \left(\frac{v}{c} \right)^5 \alpha^2 \quad (3)$$

Although we have an empirical indication of the recoil velocity αv , we must guess at v ; take $v = 0.4c$. Then

Table 1 Angular momentum and efficiency of radiation of various objects

Object	l ($\text{cm}^2 \text{s}^{-1}$)	ϵ
Neutron star rotating at breakup	10^{16}	10^{-4}
Crab pulsar $P = 0.03$ s	2×10^{14}	10^{-11}
Typical pulsar $P = 1$ s	6×10^{12}	10^{-17}
White dwarf $P = 71$ s	3×10^{16}	10^{-7}
White dwarf $P = 2 \times 10^3$ s	1×10^{15}	10^{-8}
White dwarf $P = 2$ h	3×10^{14}	10^{-10}

$$\epsilon < 10^{-11} \quad (\text{globular cluster sources}) \quad (4a)$$

$$\epsilon \approx 10^{-9} \quad (\text{galactic pulsars}) \quad (4b)$$

These estimates are disappointingly low. They also require very small values of α , in apparent disagreement with the observed masses of supernova debris. The low observed recoil velocities of the compact remnants imply that the core of the explosion is very symmetric; the moderate velocities and high masses of the exploded envelopes imply that the energy released in the core is spread over the massive envelope of the original star, slowing the velocity as a large mass is swept up. Our estimates yield kinetic energies of $< 4 \times 10^{49}$ erg and $\approx 4 \times 10^{50}$ erg from the globular cluster sources and the pulsars respectively, not far from observed supernova energies. The sweeping up of the stellar envelope occurs at much lower densities and on longer time scales than the core collapse, and hence radiates negligible gravitational radiation, despite the greater mass involved. An alternative estimate, assuming massive debris ($\alpha = \frac{1}{2}$), yields even lower values of ϵ than equation (4) because then the data require small values of v .

The preceding estimates have led to low values of ϵ because a model for the explosion was chosen in which any gravitational radiation producing asymmetry also produced a net recoil, which is strictly constrained by the data for the observed objects. There are other kinds of varying quadrupole which radiate, but which are not so constrained. Saenz and Shapiro⁵ have calculated higher efficiencies from asymmetric core bounces. Another kind of asymmetry, resulting from the flattening of a collapsing rotating object, may be empirically estimated. We have no direct knowledge of the rate of rotation of the cores of ordinary stars before their collapse, but we do know how much angular momentum some of their compact remnants have; the observed spins of white dwarfs and neutron stars may be used as a guide. Accreting compact objects in binaries are observed to rapidly spin up (and, by indirect statistical arguments, must spin down when they are unobservable), so the present spins of neutron stars in X-ray binaries give no information about their spins at birth. The spin of one accreting binary white dwarf (DQ Her) is known to be 71 s. Because of its spin-up, the present period need not reflect the spin of the stellar core before it lost its envelope; nor do we know if the evolution of an accreting white dwarf which undergoes nova outbursts ever leads to collapse. Therefore, we turn to single compact objects for information about the angular momentum of stellar cores at the times of their collapses.

Pulsars are observed with spin periods between 0.03 and several seconds. If the Crab pulsar had been born with a period much faster than its present 0.03 s, the difference in rotational kinetic energy would be much larger than the observed radiated energy, the observed kinetic energy, or the inferred energy of relativistic particles. There is no direct evidence concerning the initial periods of other pulsars. For nonmagnetic DA white dwarfs, well observed objects have spin periods of at least 30 min (ref. 6). There is no evidence for any single nonmagnetic white dwarfs with shorter periods. ZZ Ceti stars may have periods of ~ 1 d (ref. 7). Magnetic white dwarfs are known⁸ with periods of 2 hours and a day; others may have periods > 100 yr. Single white dwarfs should not have changed their spin periods

appreciably since their formation, unless the periods are many years, in which case interstellar accretion torques may be significant.

For a star flattened by rotation $Q \approx r^5 \omega^2 / G$, where ω is the spin frequency in rad s^{-1} . During collapse the specific angular momentum l is approximately conserved, and Q changes on the same time scale as the collapse. At neutron star densities where the radiation is produced, $t \approx 20 GM/c^3$. Then, using crude conventional estimates as before

$$E \approx Pt \approx \frac{Gt}{5c^5} |\ddot{Q}|^2 \approx \frac{Gt}{5c^5} \left(\frac{rl^2}{Gt^3} \right)^2 \approx \frac{r^2 l^4}{5c^5 G t^5} \quad (5)$$

For ordinary stellar cores $t \approx 10^{-4}$ s, $r \approx 10^6$ cm, and the efficiency is given by

$$\epsilon \equiv \frac{E}{Mc^2} \approx \frac{r^2 l^4}{5 G M c^7 t^5} \approx (10^{-68} \text{ s}^4 \text{ cm}^{-8}) l^4 \quad (6)$$

Table 1 gives the values of l and ϵ corresponding to various objects. The value for the neutron star rotating at breakup is within an order of magnitude of a detailed calculation⁹, indicating the adequacy of our estimates. A white dwarf with a 71-s period would have $l \approx 3 \times 10^{16} \text{ cm}^2 \text{s}^{-1}$, exceeding l for a neutron star at breakup. Angular momentum would prevent the dynamic collapse of such an object to neutron star densities, reducing ϵ far below the value corresponding to the maximally rotating neutron star; ϵ is not a monotonic function of l . The collapse stops at a radius $r \approx l^2 / GM$ on a time scale $t \approx (r^3 / GM)^{1/2}$; equation (5) then leads to

$$\epsilon \approx \frac{1}{5} \left(\frac{GM}{cl} \right)^7 \approx (5 \times 10^{108} \text{ s}^{-7} \text{ cm}^{14}) l^{-7} \quad (7)$$

The further collapse of such an object is slow, because it depends on processes of angular momentum transport. Equation (7) is used on the fourth line of Table 1. Only for a narrow range of l can ϵ approach the value for a neutron star rotating at breakup.

The values of ϵ in Table 1 are again disappointingly small, especially when one recalls that both the neutron star rotating at breakup and the 2,000-s white dwarf are hypothetical objects, and that the 71-s white dwarf is the product of unusual circumstances.

It is possible that stellar collapse is an efficient ($\epsilon \approx 10^{-3}$) source of gravitational radiation, but this is not implied by any existing data. If we try to set a semi-empirical lower limit we find $\epsilon \approx 10^{-11}$ from the rotation of the Crab pulsar, or $\epsilon \approx 10^{-10}$ from that of the white dwarf with a 2-h period. The recoil velocity of pulsars may suggest $\epsilon \approx 10^{-9}$. As pointed out by Smarr¹⁰ it is important to obtain further data on the rotation of white dwarfs and the initial spins of neutron stars.

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North Atlantic Deep Water formed by the later middle Miocene

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There is evidence that in Mesozoic and Palaeogene times both north and south subpolar areas had a mild climate^{1,2}. Unlike the present day climatic symmetry, with warm equatorial regions and ice-covered polar regions, a strong planetary asymmetry developed in the middle Miocene, when the Antarctic ice cap was established while the northern high latitudes remained unglaciated³. The symmetry was restored when a Northern Hemisphere continental ice cover was established ~3 Myr ago in the middle Pliocene⁴. These changes influenced the formation of deep water in the ocean. At present the cold, dense bottom waters originate in only two high latitude areas—the Weddell Sea producing Antarctic Bottom Water, and the Norwegian Sea, producing Norwegian Sea Overflow Water which after mixing is a major component of the North Atlantic Deep Water. The production of cold deep water in the Southern Ocean started at the Eocene-Oligocene boundary³. We present here new evidence from isotope analyses of benthic Foraminifera from DSDP site 116 indicating that the production of oxygenated deep water in the North Atlantic Ocean started in the late middle-Miocene, ~12 Myr ago.

Site 116 (57°29' N–15°55' W) is on the Rockall Plateau, a shoal area at a depth of ~1,000 m. It is separated from Ireland and the Porcupine Bank by the Rockall trough and is on the present path of Norwegian Sea Overflow Water which spills through the Faeroe sill into the North Atlantic⁵. The Rockall Plateau has a continental basement and had already subsided to a depth of >1,000 m by the end of the Oligocene. The physiography of the area has not changed during the whole Neogene⁵. It is thus an ideal place to investigate the initiation of the production of cold deep water in the Northern Hemisphere.

Isotopic analyses have been performed on benthic Foraminifera from almost every section of cores 2–18, hole 116 and cores 8–11, hole 116A. Our analytical procedures⁶ are identical to those described by Shackleton^{7,8}. Results are expressed as

$$\delta = \left[\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right] \times 10^3$$

where R is the isotopic ratio $^{18}\text{O}/^{16}\text{O}$ or $^{13}\text{C}/^{12}\text{C}$ and the standard used is the PDB standard.

In 71 samples, down to core 116–12, only aliquots composed entirely of *Cibicides* were analysed. In 22 samples, from core 116–15 to 18, mixed benthics have been used but the $\delta^{18}\text{O}$ results have been corrected by –0.23‰ and the $\delta^{13}\text{C}$ by +0.66‰ to make up for the mean difference observed in cores 116–11 and 12, where both *Cibicides* and mixed benthics have been analysed for calibration. Values will be tabulated elsewhere.

The $\delta^{18}\text{O}$ results are displayed in Fig. 1a together with those from sites 281 and 279, which are representative of the Southern Ocean, near the Antarctic Bottom Water source³. Only stratigraphic correlations^{3,5} which are independent of the isotopic record have been used. These data indicate that the main $\delta^{18}\text{O}$ events (the late early-Miocene isotopically light peak, followed by an increase throughout the middle Miocene) are synchronous at these sites. We therefore consider that the stratigraphic correlations are satisfactory down to the late early-Miocene.

The analyses at sites 279 and 281 were performed on *Uvigerina* or mixed benthics, while our site 116 analyses have been done on *Cibicides*, or corrected for departure from *Cibicides* when mixed benthics have been used. *Cibicides* has been shown^{9,10} to be isotopically lighter than *Uvigerina* when taken from the same sediment. Moreover, site 279 is >2,000 m deeper than site 116 and is at present ~2 °C cooler. These effects combine to make the $\delta^{18}\text{O}$ values at site 116 consistently ~1‰ lighter than those for the southern sites. Nevertheless, data from both regions show trends similar to those already observed in other oceans^{11–13}. We find generally low $\delta^{18}\text{O}$ values in the early Miocene sediments, up to core 116–10 (foraminiferal zones N8 and N9, ~16 Myr BP) followed by a general increase. This shift has been interpreted as corresponding to the establishment of the Antarctic ice cap^{3,14}. This increase is not monotonic; oscillations of an amplitude of as much as 1‰ occur in <10⁵ yr according to the published sedimentation rate of 2.4 cm kyr^{–1}. The values remain more stable, with a noise reduced to 0.5‰, during the late Miocene and early Pliocene (cores 116–6 to 2, 116A–11 and 10). A second much smaller increase in $\delta^{18}\text{O}$ is then registered, probably reflecting the inception of continental ice sheets in the Northern Hemisphere at ~3 Myr BP (core 116A–9). The oscillations above this level mark the Quaternary glaciations. The similarity of the $\delta^{18}\text{O}$ records at site 116 and in the other oceans during the Miocene and the early Pliocene time shows that the thermal history of the deep North Atlantic has been the same as that of the other deep water masses.

We also measured the $\delta^{13}\text{C}$ of benthic Foraminifera, which primarily reflects the $^{13}\text{C}/^{12}\text{C}$ ratio of the CO_2 dissolved in the deep water. $\delta^{13}\text{C}$ values will be modified if the rate of cycling through the exchangeable CO_2 reservoir changes. The two main causes of such a change are: (1) a modification of the volume of one part of the exchangeable CO_2 reservoir, for instance, an increase or a decrease in the continental biomass⁹. Such a modification, being extra-oceanic, must be felt equally in all oceans and the $\delta^{13}\text{C}$ must change everywhere in the same way; (2) a change in the residence time of the deep water masses: a longer residence time favours the conversion through oxidation of ^{13}C -depleted carbon from marine organic matter to dissolved bicarbonate. The longer the residence time, the lower the $\delta^{13}\text{C}$ (refs 15–17). This cannot explain a change in the $\delta^{13}\text{C}$ of the world ocean as a whole: the rate of cycling of carbon through the ocean can only be changed through a change in the turnover of the deep water masses. This in turn must have local causes, such as a change in the ratio of production of cold, dense bottom water, but such causes would have different effects on the $\delta^{13}\text{C}$ of the various ocean basins according to their distance and accessibility from the source.

Figure 1b compares the $\delta^{13}\text{C}$ for site 116 and for sites 279 and 281. Both curves begin with rather low values in the lowermost Miocene section. Towards the end of the early Miocene there is a fairly sharp increase in $\delta^{13}\text{C}$; this increase is of similar magnitude at both sites: 1.1‰ at site 279 and 1.2‰ at site 116. High values seem to persist longer in the Southern Ocean than in the North Atlantic, but this may be due to stratigraphic error in the comparison of the curves. During the middle Miocene the $\delta^{13}\text{C}$ values decrease to the previous early Miocene average at both sites.

So between the early and middle Miocene the parallelism in the trends of the $\delta^{13}\text{C}$ at both sites shows that the causes for the $\delta^{13}\text{C}$ changes were extra-oceanic. In contrast, we have no indication of a modification of the chemistry of CO_2 in the oceans, an indication that the residence time of the deep water masses remained constant.

After the late middle-Miocene (core 116–7, ~12 Myr BP) the carbon isotopic record in the North Atlantic acquires a character different from that of the South Pacific. While the $\delta^{13}\text{C}$ at site 281 continues to decrease, at site 116 there is a new increase in $\delta^{13}\text{C}$ of *Cibicides* of as much as 0.7‰ when measured on peak values. This is followed by stabilisation at values on average 0.3‰ above those of the earliest Miocene. These late Miocene $\delta^{13}\text{C}$ values are the same as those of Holocene *Cibicides* from

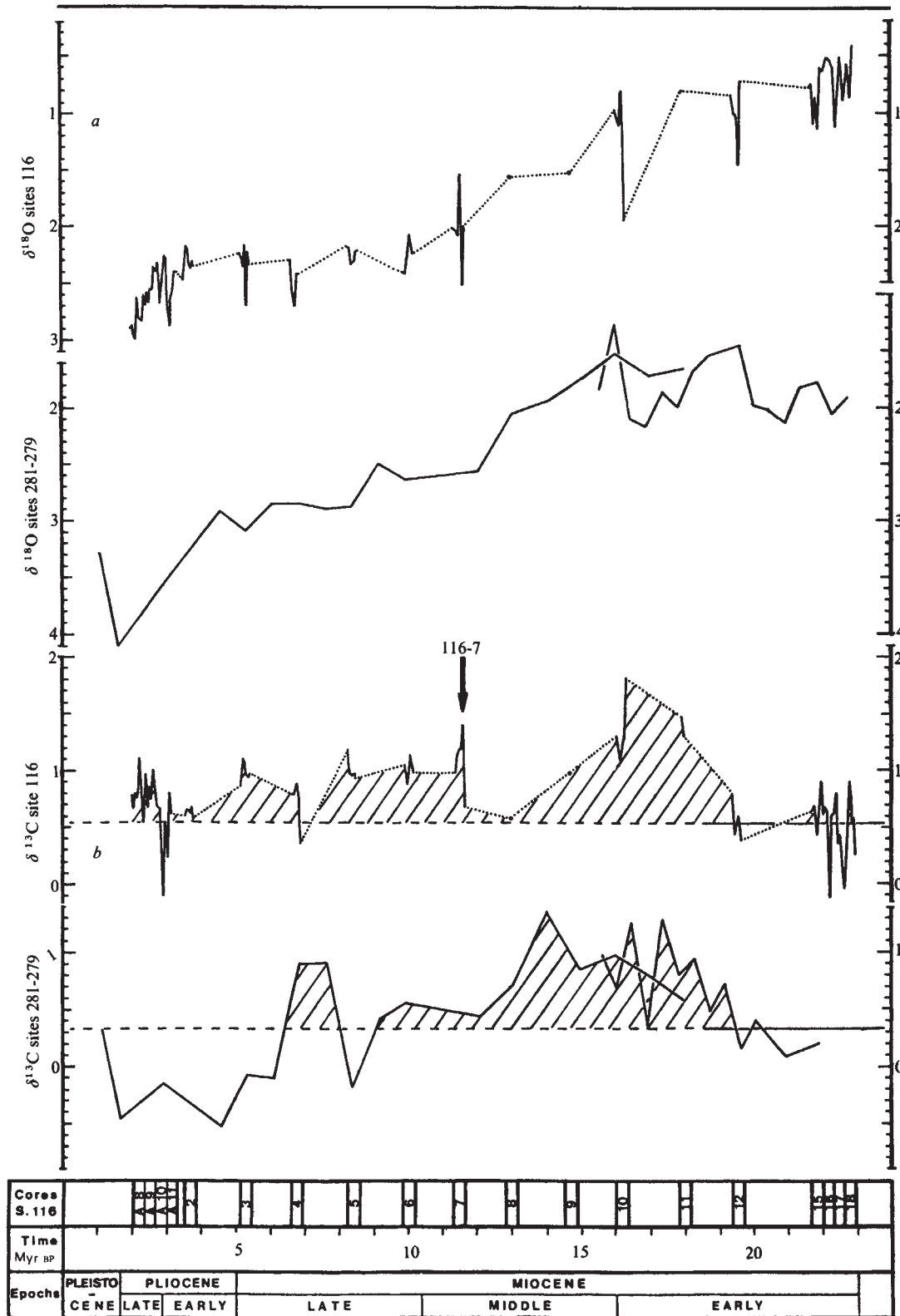


Fig. 1 Oxygen and carbon isotopic composition of benthic Foraminifera at DSDP site 116 (North Atlantic) compared to DSDP sites 279 and 281 (Southern Ocean). For comparison, a dashed line showing the average $\delta^{13}\text{C}$ for the early Miocene has been drawn throughout each ^{13}C curve and the area above this line has been shaded.

piston core CH 73139 C taken on the Feni ridge (J.-C.D., unpublished results). In the same way, at equatorial Atlantic site 366, the benthic Foraminifera, which also lived in the North Atlantic Deep Water, show $\delta^{13}\text{C}$ values similar to the present day values down to the late- to middle-Miocene boundary. Those values are $\sim 0.8\%$ below the late early-Miocene peak values, in comparison with 0.9% difference at site 116. The records are thus similar at both sites, but at site 366 a condensed sequence encompassing most of the middle Miocene precludes any accurate observations of the carbon shift¹⁸.

Thus, at ~ 12 Myr BP the deep water in the North Atlantic Ocean became significantly richer in ^{13}C than before. No such enrichment is found in the Southern Ocean which indicates that it is a local North-Atlantic event. The present residence time of the North Atlantic Deep Water is short, and as the $\delta^{13}\text{C}$ of *Cibicides* has been of the same order during the past 12 Myr then this residence time must have been short during that period. Although the amplitude of the ^{13}C -shift in core 116-7 is difficult to estimate accurately, it is of the same order as the difference presently observed between the North and South

Pacific deep waters¹⁵⁻¹⁷. This shows that the previous residence time of the North Atlantic Deep Water was high, reflecting its great distance from the Deep Water source. No cold deep water was produced in this area as the coasts of the North Atlantic Ocean, which did not yet communicate with the Norwegian-Greenland sea, still enjoyed mild climates. The southern high latitudes were then the only sources of cold bottom water. The present influence of Antarctic Bottom Water is felt as far as 40° N in the Atlantic Ocean. Until the middle Miocene, it must have been felt even further north, as it now does in the North Pacific.

We have assumed that the late middle-Miocene age for the initiation of North Atlantic Deep Water depends on the stratigraphic interpretation of the fossil record at site 116. This interpretation was made difficult by the discontinuous coring, the severe drilling disturbance and the high latitude of the site.

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Late Miocene palaeo-oceanography of the Atlantic: oxygen isotope data on planktonic and benthic Foraminifera

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The oxygen isotopic compositions of tests of Foraminifera are quantitatively related to global ice volume^{1,2} and local temperature and salinity conditions in which the Foraminifera grew³⁻⁸. Thus, a well-preserved sample of deep-sea core contains information about sea-surface temperature (shallow-dwelling planktonic Foraminifera), structure of the thermocline (deeper-dwelling planktonic Foraminifera), and bottom water temperature (benthic Foraminifera), and the global ice volume effect is also superimposed. The progress which has been made in calibration of core-top isotopic data to modern oceanographic data^{5,7,8}, has led us to test state-of-the-art core-top methodology and calibration on a late Miocene time slice (6.0 ± 0.5 Myr BP) of the Atlantic Ocean. Samples studied can be related to either uppermost Zone NN11 or lowermost NN12 of Martini⁹ and uppermost Zone N17 of Blow¹⁰.

Our experimental design was as follows. We selected core samples representing a wide range of latitude because simultaneous consideration of bottom water conditions and high-latitude and low-latitude surface conditions imposes important constraints on interpretation of isotopic data. Then we sampled tightly controlled size fractions of numerous species of both planktonic and benthic Foraminifera from each sample. By sampling numerous species, our data should include the isotopically lightest and heaviest of the planktonic Foraminifera. We evaluated possible diagenetic alteration of Foraminifera

The date of 12 Myr BP for the same event is even more tentative, as it also relies on correlations between biostratigraphic events and radiometric dates.

Still, this date is in remarkable agreement with the age assumed for the subsidence of the main platform of the Iceland-Faeroe ridge below sea level^{19,20}. The area of production of cold dense water must thus have been north of the ridge, either in the Norwegian Sea as it is today or further north in the Arctic Ocean. The appearance of this water south of the ridge is mainly a consequence of a tectonic process, and not of a climatic event, although cold winters are necessary to initiate the sinking of the surface water.

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wall structure using cathodoluminescence and scanning electron microscopy. Recrystallisation and/or precipitation of calcite after sediment deposition can seriously affect palaeo-oceanographic information. Finally, we compared analytical results with calculated values for the modern ocean and other explicit models.

Samples selected for study were washed, picked, and ultrasonically cleaned at the US Geological Survey, Menlo Park, taking care not to expose materials to high temperature or harsh chemicals. (Taxonomic identifications were done by R.Z.P.). Machine-ready Foraminifera samples were then shipped to Brown University where they were roasted *in vacuo* at 380 °C for ~1 h, reacted *in vacuo* with water-free polyphosphoric acid at 50 °C, and analysed on an on-line VG Micromass 602D mass spectrometer. PDB calibration via calcite standards from three other laboratories is within ±0.2‰ of these laboratories. Ten percent of all data were randomly duplicated with an average $\frac{1}{2}\Delta \pm 0.06\%$ for $\delta^{18}\text{O}$ and $\pm 0.08\%$ for $\delta^{13}\text{C}$. Our results are presented in Table 1 and Fig. 1. (Analytical details are available from the authors.)

Species lists in Table 1 are ranked on the basis of oxygen isotopic composition of the largest size fraction analysed, isotopically lightest (shallowest-dwelling planktonic Foraminifera) to isotopically heaviest (benthic Foraminifera closest to equilibrium). Note that although benthic $\delta^{18}\text{O}$ values in DSDP 116, 5/5, 119-121 cm are extremely light, we report these data because there is no objective reason to reject these analyses. As we did not have sufficient material to repeat the analyses we disregard all data from this sample in favour of data on 116, 5/5 143-145 cm.

The similarity between $\delta^{18}\text{O}$ species ranking in Table 1 and analogous core-top data is encouraging. High latitude sites (408 and 116) exhibit <1% variation among planktonic species whereas low latitude sites (98 and 366A) exhibit approximately 3% variation from isotopically lightest to isotopically heaviest planktonic Foraminifera. These relationships are quite similar to core-top studies^{5,8}. Further, *G. sacculifera*, *G. triloba*, *O. universa*, *G. cultrata* (s. 1.), and *G. scitula* are all well known from plankton tow and core-top^{5,7,11,12,13} and display generally similar rankings in most of our late Miocene samples.

Figure 1 compares late Miocene and modern $\delta^{18}\text{O}$ values with latitude. Late Miocene palaeo-oceanographic conditions are deduced by comparing the Miocene data with the appropriate values calculated from modern conditions⁸.

Table 1 Stable isotopic data for size fractions of various species of planktonic and benthic Foraminifera in a 6.0 ± 0.5 Myr BP transect of the Atlantic from Iceland to Rio Grande Rise

Sample	$\sigma^{18}\text{O}$ (‰, PDB)	$\delta^{13}\text{C}$ (‰, PDB)	Sample	$\delta^{18}\text{O}$ (‰, PDB)	$\delta^{13}\text{C}$ (‰, PDB)
DSDP Site 408, 63°23' N, 28°55' W, 1,624 m			DSDP Site 98, 25°23' N, 77°19' W, 2,769 m		
Core 15/5, 30–32 cm			Core 3/3, 17–21 cm		
<i>Globigerina bulloides</i> , 412 $\mu\text{m} \pm$	+1.69	−0.01	<i>Neogloboquadrina acostaensis</i> , 312 $\mu\text{m} \pm$	−0.17	−1.32
<i>Globigerina bulloides</i> , 275 $\mu\text{m} \pm$	+2.59	−0.88	<i>Neogloboquadrina acostaensis</i> , 250 $\mu\text{m} \pm$	+0.24	−1.88
<i>Neogloboquadrina atlantica</i> , 500 $\mu\text{m} \pm$	+2.30	+1.15	<i>Globorotalia scitula</i> , 437 $\mu\text{m} \pm$	+1.75	+0.05
<i>Neogloboquadrina atlantica</i> , 425 $\mu\text{m} \pm$, (2)	+2.20	+0.78	<i>Globorotalia scitula</i> , 350 $\mu\text{m} \pm$	+1.07	−0.11
<i>Globocassidulina subglobosa</i> (benthic), 350–450 μm , (2)	+3.03	+0.50	<i>Globorotalia scitula</i> , 287 $\mu\text{m} \pm$	+1.10	−0.06
DSDP Site 116, 57°30' N, 15°56' W, 1,161 m			<i>Globocassidulina subglobosa</i> (benthic), 540 $\mu\text{m} \pm$	+3.18	+0.15
Core 5/5, 119–121 cm*			DSDP Site 366A, 5°41' N, 19°51' W, 2,860 m		
<i>Globigerinita</i> sp. 375 $\mu\text{m} \pm$	+1.53	+0.67	Core 12/1, 99–101 cm		
<i>Globigerina eamesi</i> , 375 $\mu\text{m} \pm$	+2.00	−0.43	<i>Globigerinoides sacculifera</i> , 500–800 μm	−1.20	+2.76
<i>Neogloboquadrina atlantica</i> , 375 $\mu\text{m} \pm$	+2.07	+0.63	<i>Globigerinoides sacculifera</i> , 200–300 μm , (2)	−1.13	+1.36
<i>Neogloboquadrina atlantica</i> , 300 $\mu\text{m} \pm$	+1.20	+0.81	<i>Globorotalia cultrata</i> (s. 1.), 600–800 μm	−0.19	+1.59
<i>Globigerina bulloides</i> , 500 $\mu\text{m} \pm$	+2.82	+0.13	<i>Globorotalia cultrata</i> (s. 1.), 400 $\mu\text{m} \pm$	+0.46	+1.08
<i>Globigerina bulloides</i> , 375 $\mu\text{m} \pm$	+1.62	+0.02	<i>Globoquadrina altispira</i> , 600–700 μm	−0.08	+1.96
<i>Globigerina bulloides</i> , 250 $\mu\text{m} \pm$	+3.06	−0.07	<i>Globoquadrina altispira</i> , 500 $\mu\text{m} \pm$	−0.34	+2.16
<i>Cibicides kullenbergi</i> (s. 1.) (benthic), 250–425 μm	+1.07	+0.44	<i>Orbulina universa</i> , 800–1,000 μm	+0.59	+1.99
<i>Planulina wuellerstorfi</i> (benthic), 400–750 μm	+1.17	+0.71	<i>Orbulina universa</i> , 600 $\mu\text{m} \pm$	+0.55	+2.06
Core 5/5, 143–145 cm			<i>Globorotalia scitula</i> , 800–1,000 μm	+1.29	+0.73
<i>Neogloboquadrina atlantica</i> , 375 $\mu\text{m} \pm$, (2)	+0.96	+0.72	<i>Globorotalia scitula</i> , 400 $\mu\text{m} \pm$, (2)	+1.23	+0.49
<i>Globigerina bulloides</i> , 300–355 μm	+1.56	+0.25	<i>Planulina wuellerstorfi</i> (benthic), 380–750 μm	+2.43	+0.36
<i>Globigerina bulloides</i> , 212–250 μm	+0.97	−0.09	<i>Globocassidulina subglobosa</i> (benthic), 275–640 μm	+2.57	−0.56
<i>Globorotalia conomiozea</i> (s. 1.), 475 $\mu\text{m} \pm$	+1.59	+0.77	CH115–86, 30°S, 35°34' W, 2,122 m		
<i>Globorotalia conomiozea</i> 300 $\mu\text{m} \pm$	+1.35	+0.43	554 cm		
<i>Globigerina eamesi</i> , 300–355 μm	+1.75	−0.28	<i>Globigerinoides sacculifera</i> , >500 μm , (4)	+0.52	+2.60
<i>Globigerina eamesi</i> , 212–250 μm	+1.28	−0.70	<i>Globigerinoides sacculifera</i> , 425 $\mu\text{m} \pm$, (2)	+0.74	+2.20
<i>Globocassidulina subglobosa</i> (benthic), 180– 250 μm	+2.55	+0.26	<i>Globigerinoides sacculifera</i> , 287 $\mu\text{m} \pm$	+0.55	+1.95
<i>Globocassidulina subglobosa</i> (benthic) <180 μm	+2.45	−0.11	<i>Globorotalia conomiozea</i> (plexus), 520 $\mu\text{m} \pm$ (encrusted)	+1.01	+2.26
DSDP Site 335, 37°17' N, 35°12' W, 3,198 m			<i>Globorotalia conomiozea</i> (plexus), 400 $\mu\text{m} \pm$ (not encrusted)	+1.43	+1.55
Core 4/3, 79–81 cm			<i>Globorotalia conomiozea</i> (plexus), 300 $\mu\text{m} \pm$ (not encrusted)	+1.58	+1.42
<i>Globigerinoides sacculifera</i> , 300–600 μm	−0.60	+0.76	<i>Planulina wuellerstorfi</i> (benthic), 525–825 μm	+2.75	+1.53
<i>Globigerinoides triloba</i> , 300–600 μm	−0.04	+1.82	702 cm†		
<i>Globigerina nepenthes</i> , 400 $\mu\text{m} \pm$	+0.55	+0.83	<i>Globigerina nepenthes</i> , >500 μm	+0.64	+1.20
<i>Globigerina nepenthes</i> , 200–300 $\mu\text{m} \pm$	+0.59	+0.63	<i>Globigerina nepenthes</i> , 375 $\mu\text{m} \pm$	+0.55	+1.24
<i>Globorotalia scitula</i> , 300–400 μm	+0.66	−0.85	<i>Globigerina nepenthes</i> , 250–355 μm	+0.85	+1.77
<i>Globorotalia scitula</i> , 250 $\mu\text{m} \pm$	−0.31	−0.75	<i>Globigerina nepenthes</i> , <250 μm	+0.36	+0.28
<i>Orbulina universa</i> , 600 $\mu\text{m} \pm$	+1.28	+1.21	<i>Globigerinoides triloba</i> , >500 μm	+0.94	+2.12
<i>Orbulina universa</i> , 300 $\mu\text{m} \pm$, (2)	+0.61	+1.08	<i>Globigerinoides triloba</i> , 355–500 μm	+1.01	+2.42
<i>Planulina wuellerstorfi</i> (benthic), 750–900 μm	+2.49	+0.67	<i>Globigerinoides triloba</i> , 250–355 μm	+1.48	+1.79
<i>Globocassidulina subglobosa</i> (benthic), 450–700 μm	+3.34	−0.03	<i>Globigerinoides triloba</i> , <250 μm , (2)	+2.12	+1.69
<i>Globocassidulina subglobosa</i> (benthic), 375– 450 μm	+3.54	+0.03	<i>Orbulina universa</i> , >500 μm , (2)	+1.34	+2.25
DSDP Site 98, 25°32' N, 77°19' W, 2,769 m			<i>Orbulina universa</i> , 355–500 μm , (2)	+1.38	+2.25
Core 3/3, 14–16 cm			<i>Orbulina universa</i> , 250–355 μm	+1.11	+1.89
<i>Hastergerina siphonifera</i> , >500 μm	−1.48	−0.34	<i>Globoquadrina dehiscens</i> , >500 μm	+1.57	+1.68
<i>Hastergerina siphonifera</i> , <250 μm	+0.67	−0.41	<i>Globoquadrina dehiscens</i> , 355–500 μm	+1.45	+1.91
<i>Orbulina universa</i> , >500 $\mu\text{m} \pm$	−1.03	+1.72	<i>Globoquadrina dehiscens</i> , <250 μm	+3.17	+0.91
<i>Orbulina universa</i> , 375 $\mu\text{m} \pm$	−0.20	+1.59	<i>Globorotalia conomiozea</i> (plexus), >500 μm	+2.27	+1.69
<i>Orbulina universa</i> , <250 $\mu\text{m} \pm$	+0.42	+0.93	<i>Globorotalia conomiozea</i> (plexus), <250 μm	+2.85	+1.04
<i>Globigerinoides sacculifera</i> , 550 $\mu\text{m} \pm$	−0.79	+2.01	<i>Planulina wuellerstorfi</i> (benthic), 500–800 μm	+2.64	+1.18
<i>Globigerinoides triloba</i> , >500 $\mu\text{m} \pm$, (2)	−0.68	+2.26	<i>Globocassidulina subglobosa</i> (benthic), 300–550 μm	+2.99	+0.38
<i>Globigerinoides triloba</i> , 375 $\mu\text{m} \pm$	−0.31	+0.95			
<i>Globigerinoides triloba</i> , 300 $\mu\text{m} \pm$	−0.29	+0.90			
<i>Globigerinoides triloba</i> , <250 μm	+0.24	+0.61			
<i>Globigerinoides obliqua</i> , >375 μm	−0.16	+0.92			
<i>Globigerinoides obliqua</i> , <250 μm	+0.10	+0.62			
<i>Globorotalia cultrata</i> (s. 1.), 500 $\mu\text{m} \pm$, (2)	−0.01	+0.38			
<i>Globorotalia cultrata</i> (s. 1.), 400 $\mu\text{m} \pm$	−0.11	+0.46			
<i>Globorotalia scitula</i> , >350 μm	+1.69	+0.24			
<i>Globigerina nepenthes</i> , >375 μm	+1.90	+0.74			
<i>Globigerina nepenthes</i> , <250 μm	+0.47	−0.33			

Planktonic species lists are ranked on the basis of $\delta^{18}\text{O}$ of the largest size fraction.

* We disregard data on this sample in favour of data on 116, 5/5, 143–145 cm.

† We disregard data on this sample in favour of data on 554 cm; diagenesis apparent.

Four features of Fig. 1 are important for interpreting our late Miocene data. First, late Miocene $\delta^{18}\text{O}$ benthic data values for *P. wuellerstorfi* and *G. subglobosa* differ from calculated modern benthic equilibrium values by -1.0% and -0.5% , respectively. These values match observed Holocene departures of these species from equilibrium. Second, the isotopically lightest late Miocene values at each of the three North Atlantic sites (408, 116, and 335) are in remarkable agreement with calculated values for the modern summer. A similar correlation of isotopic data to summer conditions has been noted in core-top studies⁸.

Third, consider the relation of isotopically heaviest planktonic Foraminifera species to the value calculated for the modern equal density surface, $\sigma_T = 26.8$. Curry has shown this surface to be the habitat of deepest-dwelling *Globorotalia* in core-top and 18,000 yr BP materials from the Indian Ocean^{8,14}. Note the outstanding agreement in Fig. 1 between isotopically heaviest late Miocene planktonic Foraminifera and the calculated $\delta^{18}\text{O}$ values for the modern $\sigma_T = 26.8$ surface. Finally, there is a general discrepancy between isotopically lightest values at Sites 98, 366A, and CH115–86 and the calculated values for modern

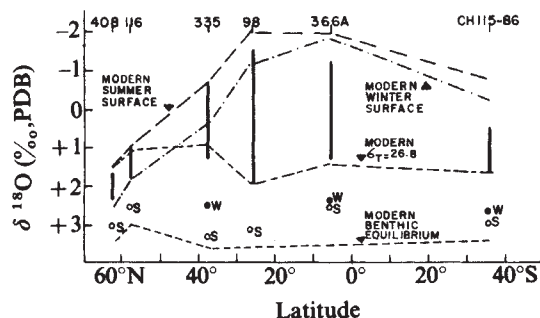


Fig. 1 Transect of the Atlantic Ocean depicting late Miocene observed and modern expected $\delta^{18}\text{O}$ values on planktonic and benthic Foraminifera plotted versus latitude. Numbers across the top are sample sites. Late Miocene $\delta^{18}\text{O}$ data on planktonic Foraminifera (bars) and benthic Foraminifera ($\circ$, *G. subglobosa*; $\bullet$, *P. wuellerstorfi*). Dashed lines represent modern expected $\delta^{18}\text{O}$ values for equilibrium with summer and winter sea-surface conditions, the 26.8 σ_T surface and bottom water. Observed late Miocene data agree well with modern expected values except for anomalously heavy surface isotopic values for Sites 98, 366A, and CH115-86. The discrepancy at CH115-86 is beyond the error of measurements and calculations and, therefore, requires explanation. See text for the three alternative hypotheses.

surface conditions. In particular the discrepancy between the data for CH115-86 and the calculated values demands an explanation.

Although three of these comparisons suggest the late Miocene Atlantic was indistinguishable from the modern, the final one contradicts this conclusion. To bring these comparisons into agreement over similarities and differences between late Miocene Atlantic and the modern, we consider three hypotheses: (1) late Miocene ice volume was larger than today; (2) site CH115-86 was significantly colder in late Miocene than it is today; and (3) the surface-dwelling Foraminifera at CH115-86 yield heavy $\delta^{18}\text{O}$ values because they have undergone significant shallow burial diagenesis.

(1) If the discrepancy between late Miocene and modern at CH115-86 is attributed to greater ice volume in late Miocene, then this effect must also be superimposed on sea-surface and benthic data for all other sites^{1,2}. The agreement between benthic data and high latitude North Atlantic surface data, and modern conditions, means that any proposal for an increased ice volume must be accompanied by a proposal of increased water temperature in those sectors. While such a proposal is not out of the question, we suggest that global ice volume, Atlantic bottom water temperatures, and North Atlantic sea surface temperatures in late Miocene were similar to modern and seek some other explanation for the discrepancy at CH115-86.

(2) If one considers that site CH115-86 really was four or five degrees colder in late Miocene than it is today, one plausible palaeo-oceanographic cause of this cooling is that surface gyre circulation was less intense in the late Miocene. This would reduce the zonal anomaly at Sites 98 and CH115-86, making both sites cooler than today. However, faunal evidence that argues against this interpretation. Planktonic Foraminifera assemblages ($>149\ \mu\text{m}$ size fraction) in modern sediments from the vicinity of CH115-86 contain ~5% of the tropical Foraminifera, *G. sacculifera*¹⁵. Counts of the $>177\ \mu\text{m}$ size fraction of CH115-86 samples show that *G. sacculifera* is at least 10% and sometimes as much as 20% of late Miocene faunal assemblages. Therefore, we assume sea surface temperatures in the vicinity of CH115-86 were not significantly cooler during the late Miocene than they are today.

(3) There is good reason to believe that the samples at CH115-86 (and those at Site 98) have undergone some shallow burial diagenesis. Both sites are situated above the modern aragonite compensation depth. If aragonite is buried with the sediment, the greater solubility of aragonite surely drives a dissolution/precipitation process through which calcite is

precipitated onto pre-existing calcite nuclei such as planktonic Foraminifera. In the simplest situation, this new calcite grows from pore fluid with oxygen isotopic composition similar to bottom water and at bottom water temperatures. Precipitation of such calcite would bring isotopic values for surface-dwelling planktonic Foraminifera closer to those observed for benthic Foraminifera. The isotopic data for sample CH115-86, 702 cm, is consistent with this hypothesis. Isotopic values for benthic Foraminifera in this sample are consistent with values obtained on CH115-86, 554 cm, yet all values of *G. triloba* in the 702 cm are significantly heavier than the values for *G. sacculifera* in the 554 cm sample, and the fine fraction *G. triloba* and *G. dehiscentis* in the 702 cm sample have isotopic values approaching those of benthic Foraminifera. Examination of the *G. sacculifera* material from the CH115-86 samples gives a clear indication of precipitation of calcite cement onto the interior wall of the foraminiferal tests as well as partial dissolution of original wall material.

Three out of four important comparisons between modern Atlantic and late Miocene Atlantic, yield indistinguishable $\delta^{18}\text{O}$ values for the two oceans. We are optimistic that stable isotope data can be used to quantify several important aspects of palaeo-oceans and to place extinct species within their three-dimensional ecological context in still older palaeo-oceans. We are convinced that diagenesis can seriously compromise the palaeo-oceanographic integrity of foraminiferal isotopic data, but that it can be recognised and avoided. Care should be taken in future studies on samples which may have been deposited above the aragonite compensation depth, and scanning electron microscopy and chemical studies should be used to demonstrate the palaeo-oceanographic integrity of materials submitted to isotopic analysis.

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Isotopic data for scleractinian corals explain their palaeotemperature uncertainties

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New data on the variability of the oxygen isotope composition of water on a coral reef reveal that there are large changes in $\delta^{18}\text{O}$ linked to high evaporation rates and precipitation. This together with variation in growth rate is sufficient to mask large water temperature variations, which otherwise would be reflected in

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the skeletal oxygen isotope ratio. The carbon isotope ratio in the skeleton, however, does vary probably as a result of growth rate. In contrast to previous studies only the most recently formed part of the skeleton was used to reduce the possibility of diagenetic additions which would distort the results.

Calcium carbonate precipitated from water by marine organisms has an oxygen isotope composition which is temperature dependent^{1,2}. This has been used for Foraminifera which secrete carbonate in equilibrium with seawater to map palaeotemperature variations through the Pleistocene³. Weber and Woodhead⁴ have shown that although corals fractionate both the carbon and oxygen isotopes, the slope of the temperature relationship is similar to that determined by Epstein *et al.*². Most corals are characterised by alternating bands of dense and less dense skeleton. The denser layer is believed to be a result of thickening of the skeletal components and a closer bundling of the aragonite crystals. Attempts to use the temperature dependence of the ¹⁸O ratio to determine in which part of the year the denser bands were formed have led to conflicting conclusions⁵⁻⁷. From isotopic evidence Weber *et al.*⁵ concluded that the bands were formed during the warmer part of the year and although similar trends were noticed by Goreau⁶ he discounted the trend as the corals which he examined formed their dense bands in the winter when the temperature is low. Emiliani *et al.*⁷ suggested that the denser bands could be produced in both summer and winter, however, they also noted a broad inverse correlation between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in contrast to other researchers who found positive correlations⁸⁻¹⁰ and Goreau who found no correlation at all⁶. In contrast to oxygen, the carbon isotope ratio may be related to growth rate. Emiliani *et al.*⁷ concluded that during the slower growth of the coral less fractionation occurred and therefore $\delta^{13}\text{C}$ approached that of seawater. However, considering other reports^{8,11} that corals growing at depth are depleted in ¹³C with respect to corals growing near the surface, this explanation apparently is not plausible, as surface corals generally have much higher growth rates¹².

The palaeotemperature equation developed by Epstein *et al.*² and modified by Craig¹³

$$T = 16.9 - 4.21(\delta c - \delta w) + 0.14(\delta c - \delta w)^2$$

shows that the oxygen isotope composition of a carbonate is not only a function of temperature but also depends on the isotope composition of the water; $\delta c - \delta w$ is the measured difference between the carbonate and water. For seawater, variations in $\delta^{18}\text{O}$ are related to changes in salinity¹⁴, but previously, local and short-term variations were considered to have a negligible effect on the $\delta^{18}\text{O}$ of skeletal carbonates. In an attempt to examine these effects on the aragonite skeletons of a scleractinian reef coral, samples of water and specimens of *Acropora cuneata* were collected in January 1978 from three sites across the reef profile at Heron Island, Australia.

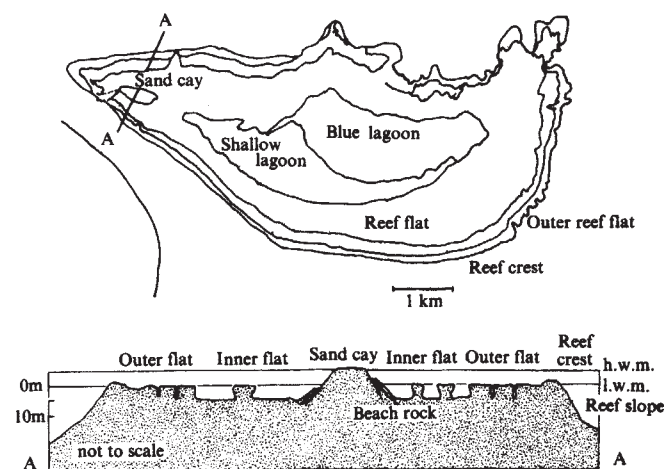


Fig. 1 The physiographic zonation and cross-section of Heron Island Reef.

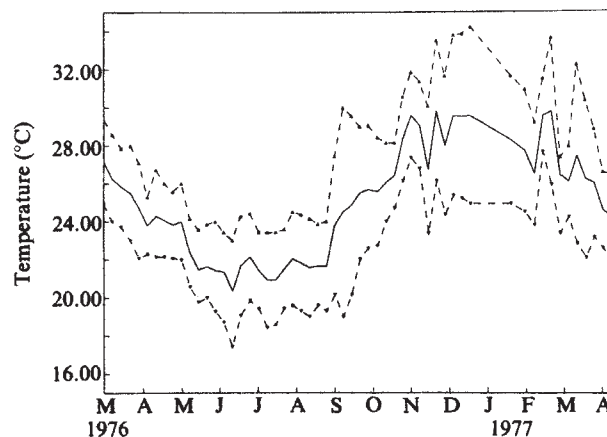


Fig. 2 Average weekly seawater temperatures on the reef flat at Heron Island, March 1976–April 1977 (unpublished data from D. Potts). The maximum and minimum temperatures are shown by the dashed lines.

Heron Island is a typical lagoonal platform reef¹⁵, near the southern end of the Great Barrier Reef, showing standard reef zonation (see Fig. 1). The reef flat has been shown to experience diurnal temperature variations as high as 12 °C (ref. 16) (Fig. 2), whereas off the reef temperature ranges are small. These variations arise at low tide, during which there may be several hours of slack water. At this time, the semi-isolated pools on the reef flat may become subjected to intense heating resulting in changes in temperature and salinity. However, changes in salinity also result from evaporation caused through high wind speeds and dilution due to rainfall. Water samples were collected over a 3-month period, mainly from the reef flat, to determine the range of salinity and $\delta^{18}\text{O}$ variation. These results are shown together with the data of Lowenstam and Epstein¹⁴ in Fig. 3.

For the carbonate isotope analysis only the terminal growth portion of each specimen was used to avoid the possibility of any diagenetic carbonate being present. Diagenetic additions occur very shortly after initial growth and their presence would distort the result. Before analysis, the organic material known to be intimately associated with the skeleton must be removed, as the organic molecules in the extracted gas interfere with the analysis: (the effects of the organic material has been summarised elsewhere¹¹). As removal of organic material with sodium hypochlorite solution or roasting in a helium atmosphere may alter the isotope ratio, the organic material was removed in a low temperature oxygen plasma furnace. Periods of up to 12 h had no effect on the isotopic composition of either control samples or the laboratory standard.

For isotopic analysis, carbon dioxide was extracted from the carbonate by the normal method, reaction with phosphoric acid¹⁷. This process involves an isotopic fractionation of oxygen which is different for calcite and aragonite (1.01025 and 1.01034, respectively¹⁸). As the samples were analysed with reference to a calcite standard, the $\delta^{18}\text{O}$ values shown in Fig. 4 were corrected for this effect (as well as the usual isobaric interferences and instrumental effects^{19,20}). Similarly, calcite and aragonite precipitated at the same temperature have different isotopic compositions^{21,22} for which a correction must be made if attempting to calculate temperature, or isotopic composition of the water. Previous papers on corals⁵⁻¹¹ have not stated whether any of these correction factors were applied.

Corals growing on the reef flat (Fig. 1) might be expected to reflect the large temperature variations as well as the changes in the $\delta^{18}\text{O}$ content of the water in their skeletons. Corals growing on the reef slope which is a much more stable environment should be isotopically more uniform. However, Fig. 4 shows that there are no discernable differences in the $\delta^{18}\text{O}$ between the three environments even though the heaviest oxygen values occur off the reef flat as one might expect if there were a

temperature relationship. As far as the $\delta^{13}\text{C}$ value is concerned, there is a statistically lower value on the reef slope, thereby confirming previous results^{8,11}. Woodhead's¹² statement that corals at Heron Island on the reef slope have a much lower growth rate than those on the reef flat supports the idea of Goreau⁶ that faster growing corals utilise more inorganic carbon relative to metabolic carbon which has more negative values.

If the present results are compared with similar data collected from the Heron Island locality by Weber and Woodhead⁸, the values for the oxygen isotope ratios are 1‰ lighter. This apparent discrepancy may be a result of preparation procedure, as Weber and Woodhead⁸ removed the organic material from their corals using the helium treatment; however, this should have made their corals lighter than ours, they are in fact heavier. Either there has been a drastic change in the prevalent environmental conditions or there are differences in the analytical techniques. Another possible source of contamination is subsequent diagenesis, either through isotopic exchange or the activities of endoliths such as fungi, algae and bacteria: boring filaments are known to infest coral skeletons within hours of it being formed²³. Clearly it is practically impossible to eliminate all subsequent diagenetic affects. Sampling the growth bands within coral heads therefore will include the products of a wide range of diagenetic processes in the sampled carbonate; this is particularly relevant to the denser bands, which coincide with green algal bands often seen when a coral head is broken open²⁴.

Isotopic data obtained from all marine organisms must be interpreted with extreme caution. There are too many uncertainties concerning the controlling processes in modern calcareous organisms, without considering subsequent diagenetic effects. The reasons for the lack of differences in the skeletal $\delta^{18}\text{O}$ value between the various habitats in the present research, despite the large temperature differences, is probably a result of either or both of the following factors: (1) The variation in the seawater $\delta^{18}\text{O}$ alone would be sufficient to mask an actual temperature difference of 7 °C; however, as the $\delta^{18}\text{O}$ of seawater may vary sympathetically with temperature, an even greater temperature difference may be masked. (2) An increase in temperature increases the metabolic processes of the coral and therefore alters the proportions of oxygen and carbon incorporated from the various organic and inorganic sources proposed by Weber and Woodhead⁸ and Goreau⁶. According to Goreau⁶ an increase in growth rate increases the isotopically heavier inorganic component incorporated. This effectively cancels out the temperature dependence of the $\delta^{18}\text{O}$ value. As the $\delta^{13}\text{C}$ is only slightly temperature dependent, this change in growth rate is reflected in the carbon isotope composition.

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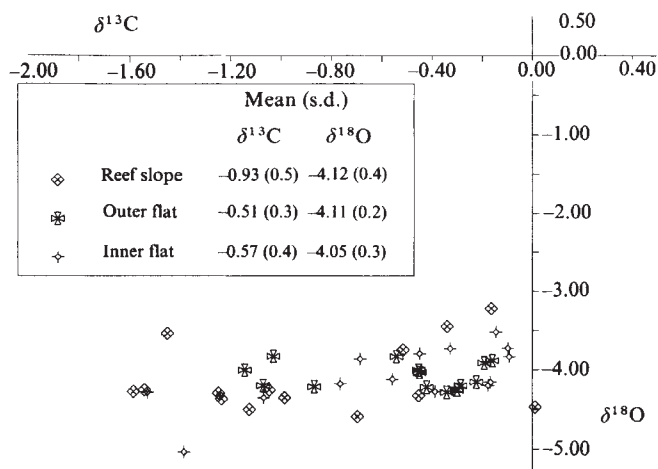


Fig. 4 Data for 45 specimens of *Acropora cuneata* collected from the inner flat, outer flat and reef slope. Values are corrected for the fractionation difference caused by dissolution in phosphoric acid, relative to calcite.

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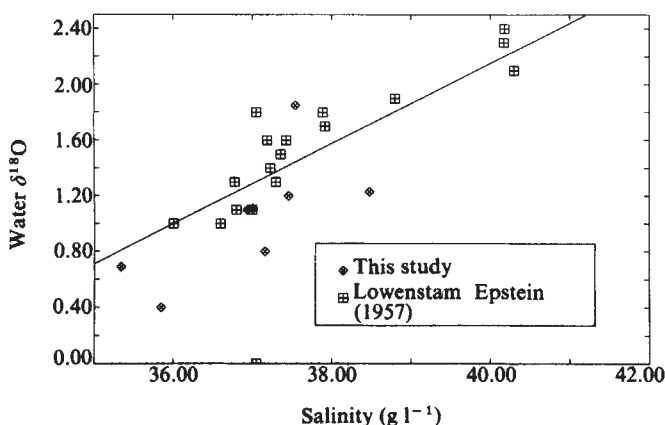


Fig. 3 Seawater $\delta^{18}\text{O}$ from Lowenstam and Epstein (1957) together with data from this study plotted against salinity. The regression line is for the data of Lowenstam and Epstein¹⁴.

Thermal effects of basalt on continental crust and crustal contamination of magmas

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A simple conduction-based heat-flow model is used to calculate the thickness of zones of continental crustal rock adjacent to basalt sheets which are raised to the temperatures of (1) hydrous mineral breakdown and (2) partial melting. Basalt emplacement leads to wide zones of partial fusion and aqueous fluid circulation at most crustal levels. Contamination of the basalt magma by partial melts or aqueous fluids is most effective where magmatism is episodic and repetitive on a 0.1–1,000 yr time scale.

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Table 1 Thermal properties and symbols

Property	Symbol	Value assumed
Distance	x	
Time	t	
Temperature	T	
<i>Crustal rocks</i>		
Density	ρ	2.7 g cm^{-3}
Heat capacity	C	$840 \text{ J kg}^{-1} \text{ K}^{-1}$
Thermal conductivity	K	$2.5 \text{ W m}^{-1} \text{ K}^{-1}$
Initial thermal gradient		15 K km^{-1} (Fig. 1)
<i>Basalt magma*</i>		
Latent heat of fusion†	L	420 kJ kg^{-1}
Liquidus T	T_{liq}	1200°C (1,473 K)
Solidus T	T_{sol}	900°C (1,173 K)
Volume fraction crystallised‡	V	

* Basalt was taken to have the same density, heat capacity and thermal conductivity as the crustal rocks; this has very little effect on the results.

† Crystallisation was assumed linear with temperature, and latent heat assumed to be liberated uniformly between liquidus and solidus temperatures.

‡ As a result of the above assumption derivative $\partial V/\partial t$ is obtained by the substitution $\partial V/\partial t = \partial T/\partial t \cdot 1/(T_{\text{liq}} - T_{\text{sol}})$ (see ref. 1).

The thermal calculations were performed using a program written by Wells¹ to solve by an implicit Crank–Nicolson finite difference method the one-dimensional equation

$$\frac{\partial T}{\partial t} = \frac{K}{\rho C} \cdot \frac{\partial^2 T}{\partial x^2} + \frac{L}{C} \cdot \frac{\partial V}{\partial t}$$

Symbols and assumed values for parameters are given in Table 1. This simplification of the full equation given in ref. 1 arises because radiogenic heat production is ignored, and the thermal conductivity treated as constant. The principles, and results, do not differ materially from those given by Jaeger², except that initial crustal temperature and its effect on the rate of heat transfer are taken into account.

Basalt sheets in any orientation are emplaced into the crust as a single pulse of magma. The heat flow equation is solved at right angles to these infinitely extensive sheets. Two thermal effects are considered:

(A) Aqueous fluid circulation in the crustal rock. Where a free fluid phase is present, it will move in response to the thermal gradient. Where there is no initial free fluid phase, H_2O movement will begin when hydrous minerals break down. The stability limits for common hydrous minerals are shown in Fig. 1. The zones of country rock raised above the breakdown temperatures of muscovite and biotite in the absence of quartz are shown in Fig. 2.

(B) Partial melting of the crustal rock. Almost all crustal rocks containing a free fluid phase can yield some partial melt at the H_2O -saturated granite minimum (Fig. 1)³. The zone of country rock heated above this curve is shown in Fig. 2. Where there is no free fluid phase, melting begins at hydrous mineral breakdown³. Therefore the probable zone of partial melting, shaded in Fig. 2, is bounded by the granite minimum to ~4 km, and by the breakdown of biotite below that depth. Movement of aqueous fluids and production of felsic partial melts must be real effects physically distinct from each other because there is a wide solubility gap between H_2O and silicate melt, spanning 70% of the compositional range even at 30 km depth⁴.

The thermal model used may yield minimum estimates for the zones of crust affected by partial melting and fluid movement because:

(1) Flow of magma through the fissure will increase the overall heat input to the country rock.

(2) The calculations are based only on heat conduction, and heat transfer by convection in the magma and by circulating fluids in the crustal rocks will speed up the heat flow.

(3) At least in the upper crust, there is likely to be a free

fluid phase. This will extend the zone of partial melting, and will be mobilised over a much greater width than the zone of hydrous mineral breakdown.

On the other hand, the estimates may be maxima because:

(4) If magmas rise through pipes rather than fissures, initial effects on crustal rocks will be unchanged, but the heated zones will later be narrower and shorter-lived.

(5) The circulation of fluids may have the effect of dissipating the heat quite rapidly, so that there is a very wide zone affected by fluids, but only a narrow partially fused zone.

(6) Many lower crustal regions may consist of granulite facies rocks, containing neither a free fluid phase nor significant hydrous minerals. Such rocks may not melt until $1,000^\circ \text{C}$, and both melting and fluid phase circulation would be essentially absent. The dotted lines below 20 km in Fig. 2 reflect this uncertainty, but it is significant that high electrical conductivity in the lower crust of two areas, both in high-grade metamorphic regions, necessitate the presence of considerable pore water^{5,6}.

(7) Absorption of latent heat of fusion by the crustal rock is not taken into account, as it is difficult to estimate the degree of partial melting. Partial fusion of 50% over a zone results in a ~30% reduction of zone width as compared to Fig. 2, while 10% partial fusion leads to only ~8% width reduction. The effects are clearly marginal to the general model discussed here.

(8) If the hydrous phase in the crustal rocks is hornblende rather than sheet silicates, the breakdown zone will be narrower than that shown in Fig. 2, and in some cases absent. Combining all these points suggests that the magnitude of the effects shown in Fig. 2 is probably realistic. The greatest uncertainty is in the lower crust.

Figure 2 emphasises the fact that in the uppermost crust, aqueous fluid circulation is the dominant effect of magma emplacement. At this level, partial melting does not occur due to the high temperature of granite minimum melting (Fig. 1). As hydrous mineral breakdown temperatures are very conservative indicators of the beginning of fluid circulation, the actual width affected by fluids in the upper 10 km will probably be at least double that shown. Norton and Taylor²⁰ have modelled such high-level effects in detail.

Figure 2 also demonstrates clearly that the basalt causes considerable partial melting of the crustal rocks at most depths. After an appropriate lapse of time, basalt sheets at 10 km depth for example, can develop partially molten zones of country rock equal to 10–20% of their own thickness. Less than 1 yr is

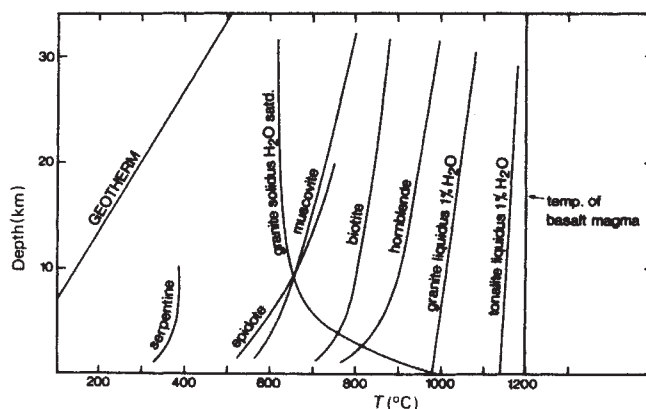


Fig. 1 The P - T equilibria relevant to emplacement of basalt magma into continental crust. The initial geotherm used is shown. Melting curves are from Wyllie³; serpentine breakdown from Johannes¹⁶; epidote breakdown under the haematite–magnetite buffer from Liou¹⁷; muscovite in the absence of quartz from Chatterjee and Johannes¹⁸; biotite (15% annite molecule, haematite–magnetite, quartz absent) from Wones and Eugster¹⁹; approximate hornblende curve from Wyllie³. Quartz absent reactions are used because there is probably insufficient time for minerals to interact; any other assumption leads to breakdown at lower temperature.

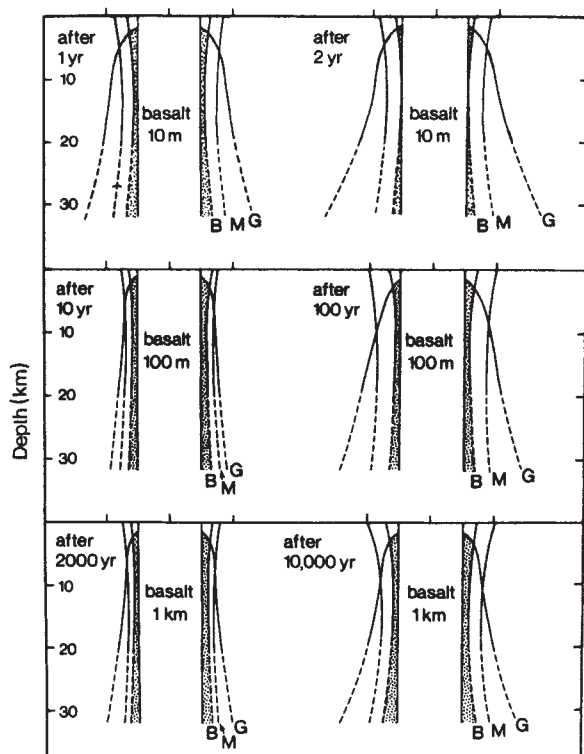


Fig. 2 The approximate temperature zones above biotite breakdown (limited by line B), muscovite breakdown (M) and the Ab-Or-Qz-V minimum melting (G) for basalt sheets 10, 100 and 1,000 m thick at 0–30 km depth. Dotted lines below 20 km reflect uncertain H₂O and hydrous mineral content of lower crust. Shaded: the probable minimum limit of partial melting adjacent to the basalt.

required for this to be achieved around a 10 m basalt sheet, 10 yr for a 100 m sheet, and as much as 2,000 yr for a 1,000 m sheet. The upper example in Fig. 2 shows that any basalt sheet ≥ 10 m thick at 10 km depth develops partially molten zones ≥ 1 m on either side after < 1 yr; clearly, some contamination of a magma by partial melts (or aqueous fluids) could take place if it flowed through its conduit for only a few weeks.

Total melting of the crust is possible only over a very short distance adjacent to the basalt, if at all. This is because while the first partial melts may be H₂O-saturated, the overall H₂O content of the crustal rock is unlikely to be $> 1\%$ (ref. 4), so that its liquidus temperature will be $> 1,000^\circ\text{C}$ (Fig. 1)³. Melts can be produced over a wide zone, but they are always partial melts.

The present study emphasises the expected widespread nature of partial melting and aqueous fluid circulation near to mafic magma conduits. These selective mechanisms (see also refs 7–9 among many others) are inherently more probable for contamination of mafic magmas than bulk assimilation (compare, for example, ref. 10). Some studies^{8,11–13} have shown that crystal-liquid or crystal-aqueous fluid isotopic disequilibrium occurs in the crust over the time scale required for contamination. This implies that the isotopic composition of partial melts and fluids generated in the crust cannot necessarily be predicted from the bulk composition of the crustal source rocks (compare ref. 14). Partial melts and aqueous fluids could easily be richer in ⁸⁷Sr for example, than the bulk crustal source rocks, because of preferential participation of Rb-rich, Sr-poor phases in the breakdown and melting reactions, and lack of time for complete fluid-residuum or melt-residuum equilibration. Such Sr diffusion data as exists¹⁵ suggests that 5 mm feldspars could remain out of isotopic equilibrium with a crustal melt for 10^3 years or more, considerably greater than the time scale envisaged for production of crustal partial melts or active fluids and their assimilation into the mafic magma. The geochemical patterns resulting from addition to the basalt magma of

contaminants which are isotopically unrepresentative of their source rocks may be highly complex. Also, as a magma may undergo contamination at several stages of its passage through the crust, no single mixing relationship is expected.

A feature of Fig. 2 is the length of time for which crustal partial melts and active aqueous fluids persist after basalt intrusion. This may be important because the major barrier to be overcome in contamination via partial melt or fluid phases is penetration through the basalt chill. In a crust which fractures to permit magma intrusion through conduits and fissures, it is unlikely that chills could remain unbroken, because further fracture must often occur after an initial magma route is established, either to admit additional dykes/conduits, or to allow increased flow through those existing. Probably the most favourable situation for contamination to occur is where magmatic pulses follow each other at intervals through the same zone of crust; say every 10–300 years for 100 m fissures. There is then a reasonable chance that new batches of magma will intrude through the heated, mechanically weakened crust adjacent to earlier largely solidified conduits, and assimilate any partial melts and hot fluids that are present. It is a common observation that igneous activity, intrusive or extrusive, is an episodic and repetitive process.

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Early development of Tethys and Jurassic ophiolite displacement

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Previous tectonic reconstructions of the Iranian segment^{1–4} of eastern Tethys have assumed that the stratigraphic positions of ophiolites from Zagros (late Cretaceous) and Makran (early Tertiary) represent the time of initial oceanic plate displacement. We present ⁴⁰Ar–³⁹Ar age spectra for mineral separates from metamorphic assemblages associated with the ophiolites of the eastern Zagros and the western Makran, Iran. One of the Zagros samples is from an amphibolite associated with metamorphosed basic and ultrabasic rocks while the other is from metamorphic olistolith within radiolarites, south of the main Zagros ophiolite body. The Makran sample is from a block of amphibolite contained within metamorphosed harzburgites.

The apparent ages suggest that initial oceanic plate disruption and tectonic emplacement of the Zagros and Makran ophiolites occurred in the middle Jurassic (~170 Myr ago) with further displacements in the late Cretaceous (~97 Myr and ~89 Myr ago). The similarity of the Jurassic age with ophiolite emplacement ages in Yugoslavia⁵ and Greece⁶ is possible evidence of a middle Jurassic collision of an early Mesozoic Tethyan ridge system with both eastern Europe and central Asia.

In the Neyriz sector of the Zagros, two distinct metamorphic regimes⁷ are separated by the Cheshmeh Anjir fault (Fig. 1). North of this fault, late Jurassic–early Cretaceous plutonism and Abukuma-type metamorphism prevail. These are probably related to ascent of thermal flux derived from a descending lithospheric plate⁷. The region south of the fault comprises thrust slices of mixed lithology that contain metamorphosed basic and ultrabasic rocks, and widespread Barrovian-type metamorphic assemblages. In a previous study of this area⁷, amphibolites associated with metamorphosed ophiolite assemblages (including spilitic pillow lavas) yielded K–Ar ages of 170 ± 8 Myr and 89 ± 7 Myr (Fig. 1). These ages were considered minimum values, and a Triassic age of metamorphism was favoured.

The ^{40}Ar – ^{39}Ar age spectrum obtained in the present study for biotite from a garnet amphibolite assemblage (H83, Table 1) is presented in Fig. 2. An apparent age plateau was not obtained for this sample. The suggestion of a low age 'saddle' in the intermediate temperature region of the spectrum is indicative of thermal alteration^{8,9}. The high temperature age, ~87 Myr, although properly interpreted as a minimum value, is probably close to the true age. The similarity between this and Adib's⁷ 89-Myr hornblende value, above, suggests that a distinct metamorphic event occurred at this time. Age spectra for biotite and muscovite from psammitic layers in an olistolith (H86, Table 1) are shown in Fig. 2b. The spectra are both essentially flat over >90% of the gas release; however, small differences in apparent age ($\pm 1\%$) are analytically distinguishable. The platform values for biotite and muscovite are respectively 98 ± 1.2 Myr and 96 ± 1.3 Myr. This suggests that a second discrete

Table 1 Sample locations and descriptions

Sample	Locality	Description
H83	Kuh-e Ceghalatun ~10 km south of Gouri, Zagros	Biotite schist layer in garnet amphibolite sequence. Overlain by recrystallised dolomitic marbles. Amphibolite cut by metamorphosed basic and ultrabasic rocks. 7 km west of 89 ± 7 hornblende K–Ar locality where spilitic pillow lavas occur ⁷ .
H86	~8 km west of Neyriz, Zagros	Olistolith of metamorphic rock in radiolarities of the Coloured Series 4 km south-west of the thrust contact of the main Zagros ophiolite body ² . Olistolith comprises bands of psammites (muscovite, biotite, epidote and piemontite layers), epidote calc-schists and epidote amphibolites.
HB1	~35 km north-east of Minab, Makran	Amphibolite in contact with serpentised harzburgites and serpentinites of Makran ophiolite. Hornblende, plagioclase with minor biotite. Displays strong lineation parallel to contact. Parallelism of fracture cleavage in serpentinites to contact suggests a thrust relationship.

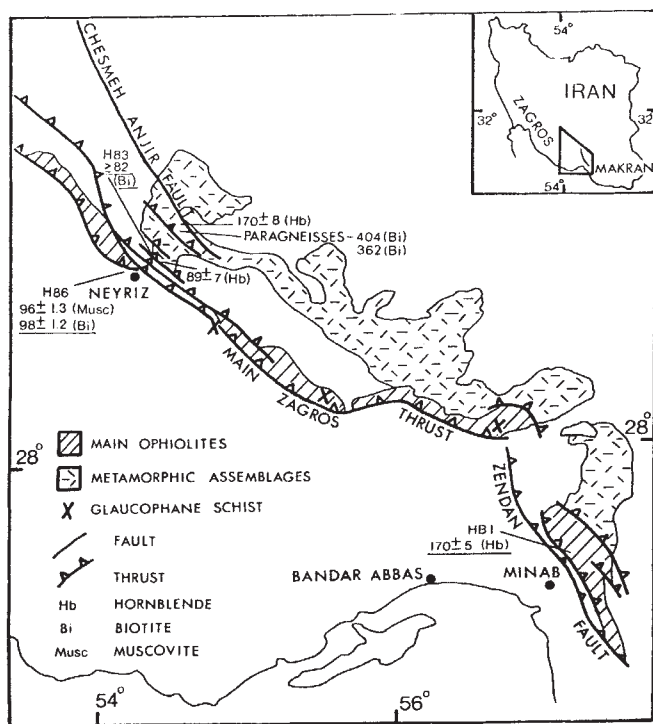


Fig. 1 ^{40}Ar – ^{39}Ar ages (underlined) with published K–Ar ages⁷ of metamorphic assemblages in ophiolite-bearing thrust slices of the eastern Zagros and western Makran.

period of Upper Cretaceous metamorphism occurred at the Iranian plate margin ~97 Myr ago. It confirms a syn- or post-Cenomanian age of emplacement for the main Zagros ophiolite, an event which may have been as late as the early Maestrichtian². Hornblende from an amphibolite enclosed by ultramafic rocks of the western Makran ophiolite (HB1, Table 1) gave a flat ^{40}Ar – ^{39}Ar age spectrum within the limits of analytical error (Fig. 2c). The plateau age, 170 ± 5 Myr, is middle Jurassic. The final emplacement age of the western Makran ophiolite is uncertain¹⁰ but Falcon¹¹ has presented evidence for late Cretaceous movements that could be related to ophiolite emplacement and exhumation. During the late Cretaceous–early Tertiary the western Makran ophiolites were displaced southwards from the Zagros along the Zandari fault (Fig. 1), which at that time may have been a transform¹². The present north-northwest structures in the western Makran (including the ophiolites) were the result of compression and overthrusting from the east along the Zandari Line in the Mio-Pliocene¹². Adib⁷ reports a K–Ar hornblende age of 170 ± 8 Myr for a garnet amphibolite from the Neyriz sector of the Zagros, immediately south of the Cheshmeh Anjir fault (Fig. 1). The agreement between this age and our hornblende age (HB1 Fig. 1) from the western Makran amphibolite indicates that a major middle Jurassic metamorphic/tectonic event affected both the Makran and the Zagros.

Tectonic emplacement models must explain the irregular distribution of amphibolites and Barrovian-type metamorphic assemblages associated with the Zagros and Makran ophiolites which are themselves deformed/metamorphosed (serpentine, rodingites, talc), and their emplacement in a sequence of high-angle thrust slices. We postulate that Barrovian type metamorphic assemblages of the Zagros and Makran result from the frictional heating/pressure effects created during descent of subducting oceanic crust beneath a continental margin; and that spatial relationships of metamorphic facies to the descending plate/continental margin are similar to those proposed by

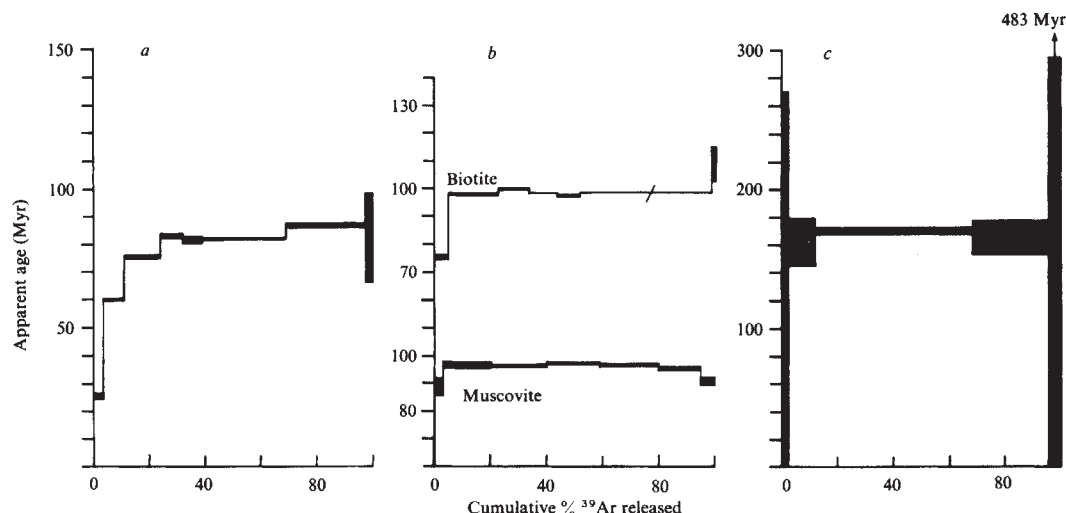


Fig. 2 Age spectra for: *a*, sample H83 biotite; *b*, H86 biotite and muscovite (the small diagonal slash separates two gas fractions); *c*, HB1 hornblende. Half-heights of black bars represent the 2σ relative (between-step) uncertainties. Decay and isotopic constants are those suggested by Steiger and Jäger¹⁴. Analytical procedures have been described elsewhere¹⁵.

Ernst¹³. Thus, the middle Jurassic (~170 Myr) ages of metamorphism represent the time of initial disruption, uplift and cooling of Barrovian assemblages associated with oceanic crust. In the eastern Zagros younger slices of disrupted oceanic plate and associated metamorphic assemblages were uncoupled and emplaced to the south probably in at least two (at ~97 Myr and ~89 Myr), and possibly more, periods during the Upper Cretaceous. Further work in the western Makran may also reveal similar discrete late Cretaceous episodes. A consequence of our model is that the ophiolite assemblages are now observed as 'cold' intrusions, thrust into supra-oceanic plate continental rise/slope sediments. Because the metamorphism occurred only at/above the subducting oceanic plate which then cooled during its ascent after disruption and uncoupling this final emplacement was terminated in the Zagros and Makran by the close of the Cretaceous period, although the position of the western Makran ophiolites seems to be modified by the Mio-Pliocene westward overthrusting along the Zendan fault. Blueschist facies metamorphism has not been reported in the Neyriz and Minab sectors but glaucophane schists⁷ are present in the most southerly (youngest?) thrust slices of the intervening area (Fig. 1). Glaucophane schists may be absent from the oldest (middle Jurassic) thrust slices because these slices contain continental basement assemblages having minimum Devonian, or older, K-Ar ages⁷ indicating that these thrusts were located on the Iranian continental margin side of Ernst's¹³ zone of blueschist metamorphism.

Amphibolites intimately associated with the ophiolites of eastern Iran, Greece⁶ and Yugoslavia⁵ all have remarkably similar (middle Jurassic) ages. We believe that this and the evidence for Permo-Triassic rifting in both Greece⁶ and south-eastern Iran^{10,16} are tectonically significant. The simplest, but not necessarily the only, explanation is to suggest essentially simultaneous middle Jurassic collision of an early Mesozoic Tethyan spreading centre (ridge) with both Europe and central Asia.

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The ecological significance of solar UV radiation on aquatic organisms

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Stratospheric ozone shields the Earth from much of the solar UV-B radiation which is the most biologically injurious component of sunlight. It is possible that both human technologies and natural events, such as reversal of the Earth's magnetic polarity, might lead to significant ozone reductions. The methodology of evaluation of the impact of an agent such as solar UV on organisms or ecosystems has not been firmly established and there has been little research in this area in the past. A new approach is proposed here. If solar UV were an important injurious factor in nature, then living organisms would be required to expend energy to cope with the solar UV exposure they receive. If organisms must specifically mitigate solar UV injury, then it would be expected that only a minimal tolerance would be achieved. We have quantitatively estimated exposure and tolerance of a number of aquatic organisms and find them to be remarkably similar.

Comparison of solar UV effects at high and low latitudes offers opportunities for analysing solar UV as an environmental agent. The visible component of sunlight is not greatly attenuated regardless of the thickness of the atmosphere penetrated, but the UV portion, especially the shorter and more biologically injurious UV-B wavelengths ($\lambda = 280-320$ nm) are strongly absorbed by stratospheric ozone and thus will be highly dependent on the solar angle. Summer solar irradiance reaching Reykjavik ($\approx 650 \text{ cal cm}^{-2} \text{ deg}^{-1}$) approximates the energy reaching temperate or tropical regions. The UV-B radiation at Reykjavik, however, is about half the maximum daily amount reaching temperate and about 40% of that reaching tropical areas. Seasonal effects on UV-B are also much greater at high latitudes.

Fig. 1 Typical exposures of marine organisms in the euphotic zone. The average UV-B exposure to the euphotic zone on a sunny summer day, assuming 10 SU incident at the water surface, as measured by the Robertson sensor^{3,4}; exposure is plotted as a function of average productivity of the station. Although UV-B transmission varied from station to station, the corresponding changes in visible light transmission tended to maintain a relatively constant exposure in the euphotic zone. Organisms in the more productive euphotic zones receive a somewhat higher UV-B exposure than at stations of low productivity.

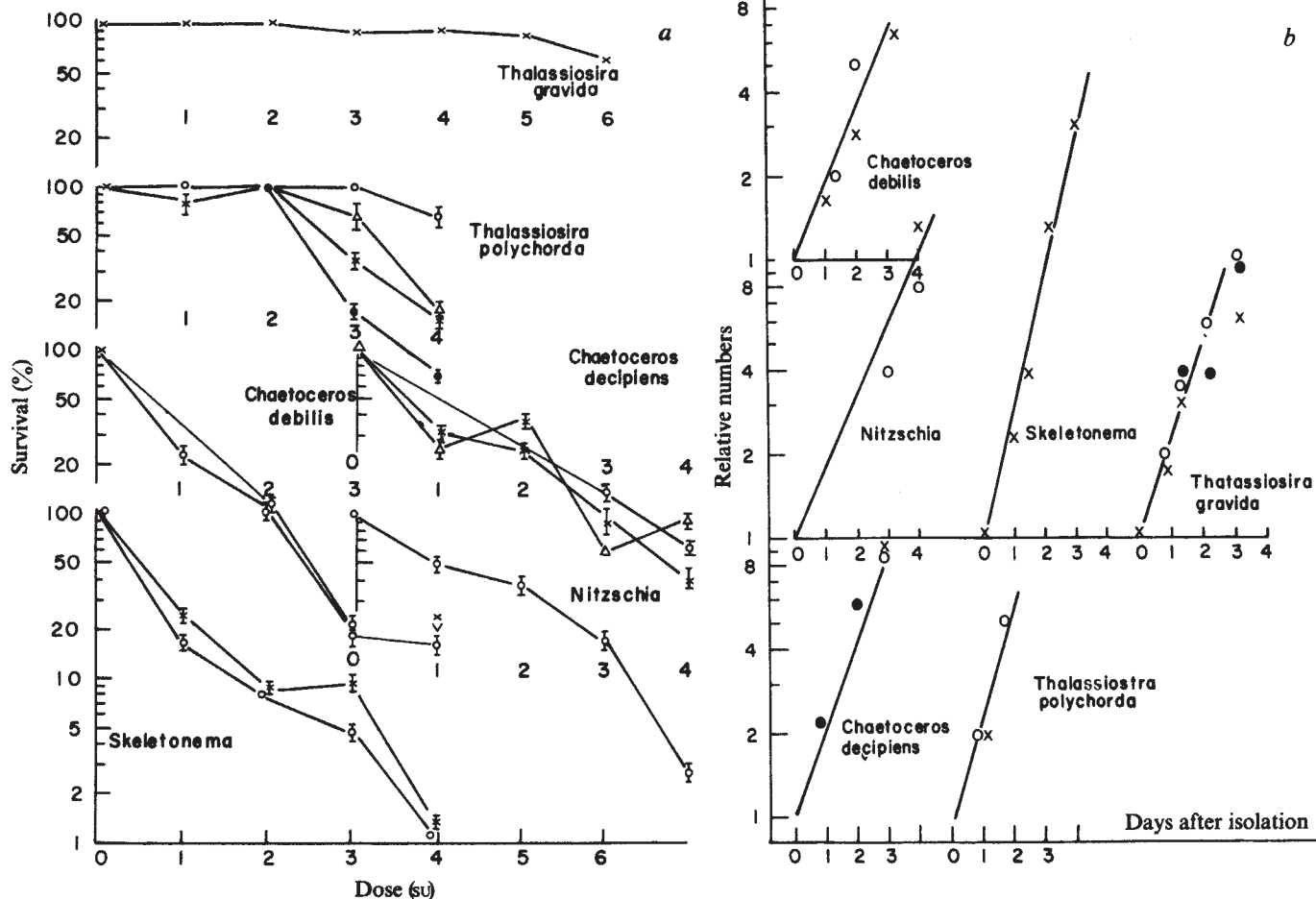
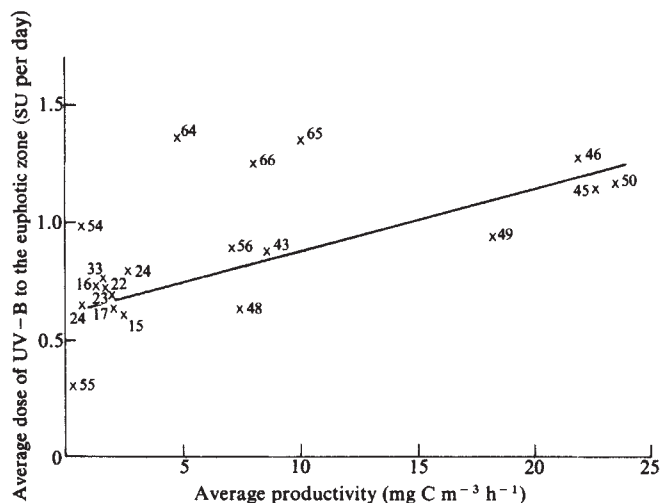


Fig. 2 *a*, The UV-B sensitivity of six marine diatoms. Components of the marine phytoplankton, six common diatoms, were selected as critical organisms for the study of biological response to UV-B. The diatoms were isolated from water samples collected in 1976. *Chaetoceros debilis* and *Chaetoceros decipiens* were derived from collections off the north of Iceland, *Thalassiosira polychorda*, *T. gravida* and *Skeletonema* were isolated from samples collected near Reykjavik. The diatoms grew well as clones in Erdschreibers medium diluted 4:1 with seawater; growth was at 7 °C under continuous illumination (1,550 lx) from fluorescent lights. Irradiated and control organisms were isolated into individual depressions containing about 0.25 ml of medium using a micropipette while observing through a low power microscope. The various species of diatoms grow in short connected chains; since the cells of these chains have no protoplasmic connections, but are only linked through inert fibres, we assume that each cell is an independent unit. The various organisms were irradiated with 'simulated' solar UV-B, generated by Westinghouse type FS 20 lamps, and filtered with a Pyrex glass filter of nominal thickness 1/8th inch (ref. 2), monitored using the Robertson sensor; the irradiance from our simulator was 2 SU h⁻¹ which approximates the highest irradiance measure in Iceland (1.67 SU h⁻¹). The test organisms were irradiated suspended in medium contained in a small cooled amber plastic dish 2 mm deep, a separate vessel was removed for each dosage and the organisms isolated after the desired exposure times. Two types of controls were run, the 'dark control' was held in a refrigerated incubator at 10 °C in the dark for the time of the longest irradiation (2 h) while a second control, the 'light control', was covered with thick plate glass, thus being exposed to identical conditions except for the UV-B component which was removed by the glass. In all cases the light and dark controls were quite similar in response. The fate of all isolates was followed until the isolated individuals had either died or shown a capacity for sustained growth. A minimum of four post-irradiation divisions was considered to be required to indicate survival. Single cell survival, accounting for control mortality and for multiple organisms in each depression, was computed along the lines previously described⁸. *b*, The growth of marine diatoms. It is assumed that injured populations recover by the growth (division) of survivors, which was determined by repeated counting of isolated diatoms, both unirradiated control organisms and organisms irradiated to various dose levels.

The effects of solar UV-B can be assessed through four independently measurable factors: (1) the amount of solar UV-B incident at the water surface; (2) its attenuation in reaching the position of the various critical organisms; (3) the UV-B sensitivity of the various species comprising the ecosystem; and (4) the capacity of the irradiated organisms to repair injury and replace components of the population killed by UV-B exposure. The four factors have been observed for Icelandic marine ecosystems and compared with similar observations made in Kentucky.^{1,2}

As expected, the UV-B irradiance incident on Reykjavik, Iceland (expressed in a biologically weighted unit, the SU)^{3,4} reached a peak near midsummer noon (high solar angles), whereas total irradiance was much less dependent on solar angle.

The attenuation of UV-B and visible light was measured at 33 locations in the oceans off Iceland. The productivity of waters from depths of 0, 10, 20 and 30 m was also determined (as ¹⁴C incorporation under standard illumination) at many of the stations.

The average irradiance incident in a given volume of water can be calculated using the Morowitz formula⁵. Using the common approximation that the euphotic zone (where photosynthesis > respiration) lies above the depth (Z_{ez}) receiving 1% of the surface irradiance, then the average UV-B dosage to the euphotic zone (during a sunny summer day) can be computed. Figure 1 shows the average UV-B dosage to the euphotic zone plotted against productivity.

The action of UV-B exposures of 0.5–1.5 SU (Fig. 1) can be established by examining the effect of such exposures on key organisms in the marine ecosystem. Figure 2a shows the survival response of the six diatom species irradiated with simulated solar UV-B. While *Thalassiosira* spp. were relatively resistant, the other four diatoms showed substantial killing at a radiation dose of 1 SU.

The maximum tolerable exposure to various agents for organisms multiplying asexually can be estimated² by determining the replacement limiting dose (RLD) for a species, which is the daily exposure which would kill a fraction of the population just equalling the daily growth capacity of the survivors.

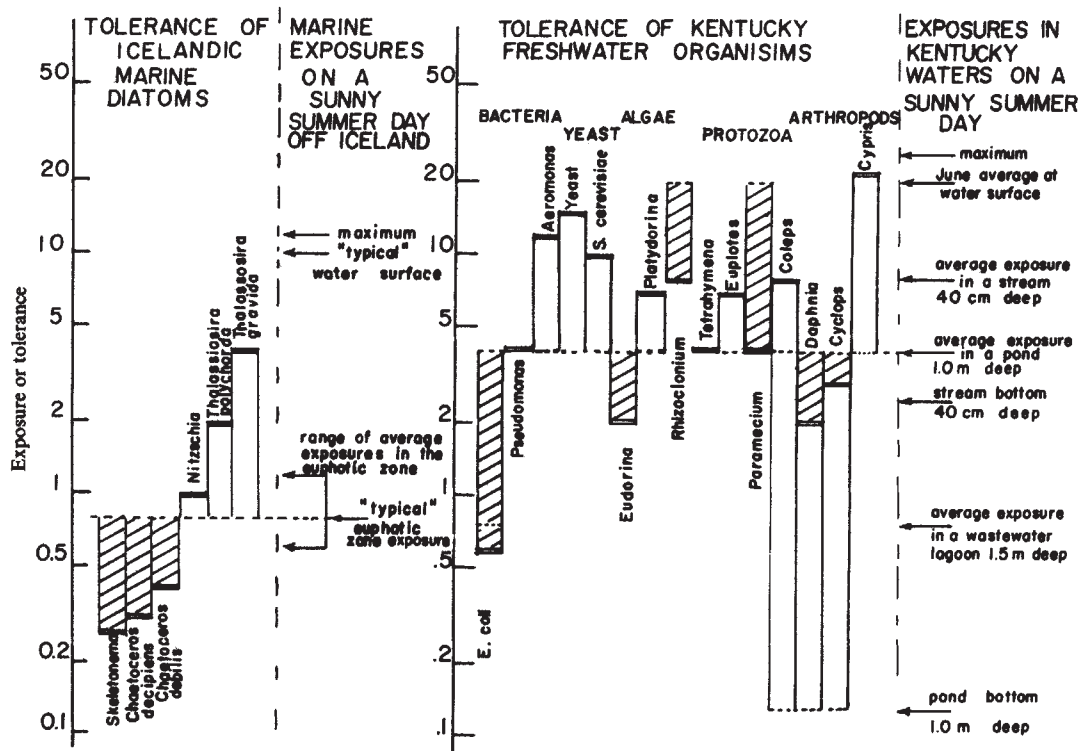
Figure 3 shows the RLD values of various organisms, together with the exposure which they might typically receive. Computed exposure often equals or exceeds the tolerance of organisms;

however, the ability of organisms to survive such an apparent excess exposure can be explained on a case by case basis. *Escherichia coli* shows a RLD less than exposure¹, although it is known to have three separate pathways of DNA repair, all of which reduce its sensitivity to sunlight⁶. It might seem that *E. coli* needs to be able to withstand sunlight; however, *E. coli* is well known to 'die off' in natural waters. Because *E. coli* spends most of its life cycle in the sunlight-protected intestine of host animals, species propagation may require short-term survival in natural waters, living only long enough to transfer from host to host.

Some protozoans and arthropods show apparent tolerance levels below the exposure levels in shallow waters (Fig. 3). *Paramecium aurelia* in particular tends to remain at the surface; however, it also shows a preference for shielded surface locations⁷ (such as rocks and plants protruding from the water). Another protozoan, *Coleps*, shows a pronounced avoidance of light by moving deep into the water column⁷. Arthropods, *Cyclops*, *Cypris* and *Daphnia*, have also been investigated. *Daphnia* can reach reproductive maturity 10–15 days after birth and produce broods of about five offspring at 10-day intervals thus showing a growth rate of 1.38 per day. RLD would equal 72% survival or 2 SU per day. *Daphnia*, *Cyclops*, and the sensitive protozoa show avoidance of UV-B exposure, coping by behaviour, while the very radiation resistant *Cypris* (RLD > 20 SU) seems to seek intense illumination⁷.

Some algae also seem to fall short of the required minimum tolerance. One filamentous freshwater alga, *Rhizoclonium*, floats on ponds during the summer and would seem to require tolerance greater than the 8 SU observed. The surface mat was heavily pigmented and senescent while new growth extended downwards and was protected by the surface layer. This organism seems to reach the required UV-B resistance in the same way as many higher organisms, through specialised protective organs or tissues. Although we lack quantitative data to explain the sensitivity of marine diatoms, they change buoyancy in response to illumination, rising with visible light and falling after UV-B exposure. It is possible that they and unicellular freshwater algae obtain adequate photosynthetic light with minimal UV-B exposure by rising in early morning and late evening and sinking for UV-B protection at midday when UV exposure is greatest, a movement pattern often observed in algae.

Fig. 3 A comparison of exposure and RLD. Maximum tolerable doses (double bars) are shown for a number of aquatic organisms together with the level of exposure for typical locations where the organisms are found. When exposure is less than tolerance the difference is indicated by solid bars; when exposure exceeds tolerance the difference is indicated by crosshatching. The apparently paradoxical situations where organisms lack sufficient resistance to live in the areas where they are, in fact, found is considered on a case by case basis in the text.



Our findings may be compared with similar calculations made for environmental ionising radiation. The RLD for 'wild-type' *E. coli* is 5–30 krad per day, almost 10^8 times the natural exposure (0.1 rad per yr); for *Tetrahymena pyriformis* the ionising radiation RLD is more than 10^9 times exposure. The ionising radiation resistance of aquatic microorganisms can hardly be an adaptation to a direct stress; resistance to ionising radiation depends on genetic characteristics which could be lost through mutation; resistance to ionising radiation must be a by-product of other requirements, perhaps resistance to solar UV, since the two forms of radiation resistance are highly correlated.

The finding that tolerance and exposure to solar UV are approximately equal strongly implicates solar UV as a significant ecological factor, suggesting that there may be no large reserve of resistance which could cope with altered solar UV exposure without requiring modification of physiology or behaviour. Doubtless most organisms would adapt if the solar UV were to increase. Organisms can increase pigmentation, repair capacity, or avoid solar UV; however, both physiological and behavioural adaptation to solar UV reduces the resources available for other purposes.

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LH-RH in the mesencephalic central grey can potentiate lordosis reflex of female rats

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The lordosis reflex, which characterises female reproductive behaviour in rodents, can be potentiated in oestrogen-primed ovariectomised female rats by electrical stimulation of the ventromedial nucleus of the hypothalamus (VMN)¹. Subsequent demonstration of an immediate and large facilitation of the lordosis reflex from the mesencephalic central grey (CG)² prompted us to speculate that the VMN may exert an oestrogen-dependent tonic bias on the mesencephalic circuitry for lordosis via its heavy descending projections³. Moreover, we considered that luteinising hormone-releasing hormone (LH-RH) could be involved in this system. LH-RH can potentiate lordosis when given systemically to oestrogen-primed ovariectomised rats^{4,5}, and neurotropic effects of this peptide have been shown by microiontophoresis^{6,7}. Also, some axons in the CG have been stained with antiserum to LH-RH⁸. The present study examined a possible role of LH-RH in the CG in the regulation of the lordosis reflex of oestrogen-primed ovariectomised female rats. Infusion of exogenous LH-RH in the CG had an immediate facilitative effect on the lordosis reflex, whereas passive immunisation against endogenous LH-RH by anti-LH-RH γ -globulin diminished the reflex.

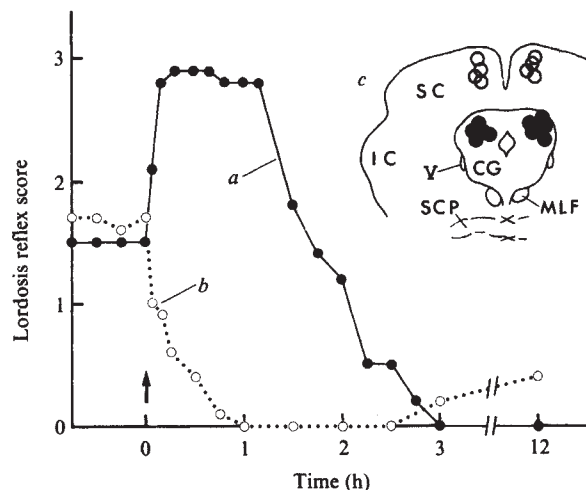


Fig. 1 Successful modification of lordosis reflex performance by LH-RH (a, ●) and anti-LHRHG (b, ○) in rat no. 6. Infusions were made at 0 h (arrow). c, Effective sites of infusions in five rats (●) were in the central grey (CG). Infusions in the superior colliculus (SC, ○) in four rats were ineffective. The diagram was taken from a frontal section of the mesencephalon 6.0 mm posterior to the bregma. Abbreviations: IC, inferior colliculus; MLF, medial longitudinal fascicle; SCP, superior cerebellar peduncle; V, mesencephalic nucleus of the trigeminal nerve.

Intracerebral infusion was accomplished in ovariectomised Sprague-Dawley female rats chronically implanted with stainless steel 22-gauge guide cannulae bilaterally in the CG. The animals received a subcutaneous injection of 5 μ g of oestradiol benzoate in sesame oil. They were given a screening test 96 h later, and those which exhibited moderate lordosis in response to manual cutaneous stimulation of the flanks followed by rump-tail base-perineum region⁹ were selected. The infusion was made with internal cannulae: 28-gauge stainless steel tubes fitted into the guide cannulae and protruding 0.5 mm from the tip of the latter. LH-RH (Ayerst) was dissolved in saline in a concentration of 100 μ g ml⁻¹. Lyophilised sheep anti-LH-RH γ -globulin (anti-LHRHG) and normal γ -globulin (NSG) were dissolved in saline to reconstruct the original concentration in the serum. Each solution was prepared immediately before use, and was infused over a period of 60 s, in a volume of 0.5 μ l through each cannula. Two sets of inner cannulae and microsyringes were used for consecutive delivery of the fluid into the bilateral cannulae. Placement of the internal cannulae and the infusion were carried out with the animal in a restrainer but not anaesthetised. The lordosis performance of an animal at a given time was assessed by applying manual cutaneous stimuli five times, at brief intervals. Lordosis reflex strength with each stimulus application was rated using a scale from 0 (no vertebral dorsiflexion) to 3 (strongest possible response), and the average of five ratings was calculated. This average, which we termed the lordosis reflex score, provided virtually identical results when collected independently by two investigators. It has been confirmed that animals with a high reflex score consistently show high receptivity with male rats^{1,2}. The lordosis score was usually determined at intervals of 5–30 min during the experiment. Following the completion of observations, tip positions of the internal cannulae were determined in frozen serial sections (100 μ m) stained with luxol fast blue and cresyl violet.

Injection of LH-RH into the dorsal part of the CG produced a prompt facilitation of lordosis which was comparable to the effect of electrical stimulation². For example, significant effects could be repeatedly produced with similar time courses in five animals. Facilitation caused by these infusions occurred within 5 min of the end of each application and lasted for about 2 h in each case. Figure 1a shows an experiment depicting the time course of the facilitation. This facilitation was followed by prolonged suppression of the reflex in three of the five rats.

Increased grooming and licking behaviour often accompanied the facilitation of lordosis, and mere brief contact on the fur of the flanks was sufficient to trigger lordosis in these animals. An equivalent volume of physiological saline had no significant effect when infused into the CG, and infusion of LH-RH into the cerebral cortex also failed to produce any effect.

Passive immunisation with anti-LHRHG resulted in a complete loss of the lordosis reflex when infused into the CG of the same animals. The reflex disappeared within 90 min after the infusion. The suppression of the lordosis reflex persisted for as long as 12 h (Fig. 1b). The lack of aggressive behaviour (often elicited in non-receptive female rodents by somatosensory stimuli¹⁰) was noted when these animals were made unresponsive by anti-LHRHG. NSG injection in the CG had no apparent effect on the performance of lordosis.

Effective sites for the infusions of LH-RH and anti-LHRHG were located in the dorsal half of the CG. Neither LH-RH nor anti-LHRHG had an appreciable effect on the lordosis reflex when infused in the superior colliculus (Fig. 1c).

LH-RH bioactivity has been reported to be present in the mesencephalon of the dog and rabbit¹¹. By immunohistochemical techniques, some axons in the CG⁸ of the guinea pig stain with antiserum to LH-RH. These extra-hypothalamic LH-RH axons have now been implicated in the control of reproductive behaviour. Although a neurotropic effect of LH-RH has not been shown for CG neurones, neurones in the medial basal hypothalamus are either excited or inhibited by microiontophoresis of LH-RH^{6,7}. Because electrical stimulation of the CG effectively facilitates the lordosis reflex in oestrogen-primed ovariectomised female rats², it is possible that endogenous LH-RH has excitatory effects on the CG neurones involved in the control of lordosis. The origin of mesencephalic LH-RH axons has not been identified, but a contribution of cells in or near VMN cannot be ruled out. LH-RH activity has been detected in the lateral part of the VMN¹². If this is the case, the requirement for oestrogen in the successful electrical facilitation of lordosis from VMN¹ may be explained at least in part by a requirement for hypothalamic LH-RH¹³, which can be mobilised by electrical stimulation. In this context, it may be significant that both LH-RH infusion and VMN stimulation¹ caused long-lasting facilitation of lordosis after each manipulation.

Earlier attempts using infusions of LH-RH¹⁴ or anti-LHRHG¹⁵ to the brain suggested modulatory effects of these substances on the performance of lordosis. With combined observations on the effects of LH-RH and anti-LHRHG, we have now demonstrated that a hypothalamic hormone is necessary for a given behaviour. Blocking the action of decapeptide LH-RH could block lordosis behaviour. In turn, an exact site in the brain where LH-RH is required, the midbrain central grey, was revealed by the location of the microinfusion cannula tips. The identification of a specific neurochemical (LH-RH) needed for reproductive behaviour and a particular site of action may allow specific interference with mating behaviour, with other behavioural responses left undisturbed.

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Paternal investment of urates in cockroaches

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During copulation, the male German cockroach [*Blattella germanica* (L.)] deposits a spermatophore in the female's genital pouch and voids stored urates from his accessory glands (uricose glands) on to the spermatophore¹. About 24 h after mating, the female expels the empty spermatophore with adhering urates. Although uricose glands have been reported in the Blattaria^{2,3}, and are proposed to act in excretion^{4,5}, their role in reproduction is unclear. Roth² has suggested that urates may serve to protect the spermatophore from premature consumption by the female or other insects. However, we have noted the disappearance of spermatophores/urates from cages containing mated pairs of cockroaches and have investigated the possibility that these materials have nutritional value. We observed that spermatophores and urates disappeared between 7 and 18 d after females were mated while on a dog food diet; spermatophores were consumed within 4 d by females starved for 3 d after eclosion. Furthermore, we now report that labelled uric acid assimilated by males before, and voided at, mating can be recovered in mated females and their ootheca.

Accessory glands containing high levels of labelled urates were obtained by injecting males with [8-¹⁴C]hypoxanthine (Table 1). One of each labelled pair of males mated successfully and after 38 d all females and their oothecae were radioactive. Table 1 shows that females on a low protein (5%) diet

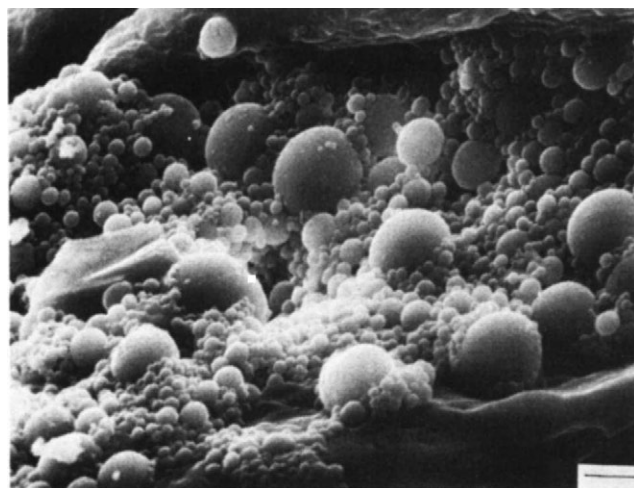


Fig. 1 A scanning electron micrograph (SEM) of the edge of an emptied spermatophore with adhering urate spherules which are secreted/deposited by males at the time of mating. These urates may be consumed (along with the spermatophore) and used by the female and/or be subsequently incorporated into the ootheca as a nitrogen resource. Before viewing, the specimen was coated with a 60:40 mixture of gold/palladium. It was examined with an Advanced Metals Research SEM (Model 900–52) equipped with EDAX (energy dispersive X-ray analysis, Model 707A). Scale bar, 5 μ m.

Table 1 Fate of labelled ^{14}C -hypoxanthine injected into young adult male *B. germanica* which were subsequently presented to young adult females

Diet	Females	Whole insect/oothecal activity in (d.p.m.)		
		Oothecae	Mated males*	Unmated males
5% Protein	2,073 $\pm$ 961 a†	9,417 $\pm$ 2,725 b	51,681 $\pm$ 9,181 c	103,948 $\pm$ 3,688 d
25% Protein	2,486 $\pm$ 704 e	800 $\pm$ 275 f	70,635 $\pm$ 4,256 g	107,746 $\pm$ 3,905 h

Adult males (1–3 days old reared on dog food) were injected with 1 μl [$8\text{--}^{14}\text{C}$]hypoxanthine, resulting in a measured 181,418 $\pm$ 6,697 d.p.m. per insect. They were maintained on 25% casein protein diet for 10 days, followed by presentation of two males (see below) to one young virgin female (reared on dog food) in a plastic Petri dish supplied either with a 5% protein or a 25% casein protein diet and water. In addition, unmated males (maintained on 25% casein protein) were frozen 10 and 48 days after isotope injection. Stock cultures, rearing conditions and diet formulations used are described elsewhere^{12,16,17}. Before extraction, insects and oothecae were rinsed several times in 0.6% Li_2CO_3 at 30 °C to remove any labelled material adhering to their exteriors. Whole insect assay included homogenising whole bodies in 0.6% Li_2CO_3 at 80 °C for 10 min, centrifugation at 1,000g for 10 min and counting in PCS (Amersham/Searle)/xylene scintillation cocktail. Oothecae were extracted in 0.1 M Tris-HCl, buffer pH 7.4, at 30 °C, centrifuged and counted as above. A portion of these extracts was frozen for subsequent analysis (Table 2). Data represent the mean $\pm$ s.e.m. ($n = 8$ females per diet). Ten days after injection virgin males contained 124,279 $\pm$ 8,443 d.p.m. per insect and after 48 days they contained 107,908 $\pm$ 2,203 d.p.m. per insect ($n = 4$). Accessory glands contained 57,989 $\pm$ 14,066 and 36,365 $\pm$ 1,644 d.p.m. per mg ^{14}C -urate in tissue obtained 10 and 48 days after injection, respectively ($n = 4$). Purification of these extracts indicated that virtually all the activity in this tissue could be attributed to labelled urate. One discarded spermatophore/urate obtained from a mated pair contained a total activity of 54,976 d.p.m. in the extracted urate fraction. ^{14}C -hypoxanthine was used instead of xanthine or uric acid because of its greater solubility in aqueous solutions; this facilitated the process of injecting relatively constant amounts in each insect. The subsequent conversion of hypoxanthine to uric acid stored as urates in the males proved to be quite suitable for our purposes.

* Virgin females were provided with two males to allow an optimal opportunity for mating to occur. In these conditions only one male will normally mate successfully¹⁸, presumably releasing its urate stores. We have arbitrarily segregated the males on the basis of whole body activity, hence 'mated males' were compared with 'unmated males'. These differences are highly significant (see below).

† Statistical analysis using the Student's t -test indicated the following: $a = e$, $P < 0.7321$; $b \neq f$, $P < 0.0152$; $c \neq g$, $P < 0.082$; $d = h$, $P < 0.4868$; $c \neq d$, $P < 0.0001$; $g \neq h$, $P < 0.0001$.

incorporated more activity into their oothecae than did those maintained on 25% casein protein diet. The amount of label consumed and transferred to the ootheca varied considerably. Presumably, this variation reflected the amount of label excreted by males, the feeding activity and nutritional status of females. Obviously, these radioactivity transfer estimates could have been influenced by metabolism of urates to $^{14}\text{CO}_2$, which was not measured.

The disappearance of deposited spermatophores/urates suggests that the major route of urate entry into females is the alimentary canal. This does not preclude the possibility that a small fraction may enter by the genital tract⁶. Radioactivity within oothecae was mainly in the soluble fraction, with very little in the precipitated protein or oothecal cases (Table 2). Analysis of the soluble fractions indicated that the major radioactive component was uric acid. Urate content was examined in oothecae at different stages of embryonic development and newly hatched nymphs. Newly formed oothecae contained 2.8 ± 0.1 μmol uric acid and newly hatched 1st instar nymphs plus their empty oothecal cases contained 1.2 ± 0.1 μmol . Correlation analysis showed a significant decline in uric acid levels during embryogenesis ($y = -0.056x + 2.72$; Pearson's $\rho = -0.789$; $P < 0.0001$). This decline strongly suggests that uric acid is metabolised during embryonic development.

Large nutritional demands are made on the metabolism of the female German cockroach during oothecal production. Kunkel⁷ has shown that the female incorporates almost 90% of her food reserves accumulated during the preovipositional period into a single ootheca. A portion of these reserves are nitrogen-containing materials which are transferred to the oocyte in the form of vitellogenin, appearing in the female blood 3 or 4 days after adult ecdysis and disappearing soon after terminal oocytes have been released into the ootheca⁸. It now seems that females are also incorporating uric acid into developing oocytes.

Because uric acid levels decrease significantly as the embryo develops, it is likely that this incorporated uric acid is metabolised during embryogenesis.

Another event occurring during oogenesis is trans-ovarian transmission of symbiotic bacteroids (mycetocyte bacteroids) from females to the oocytes⁹. Although the precise role of cockroach mycetocyte bacteroids is not clearly understood, they have been implicated in amino acid, urate and vitamin metabolism^{9–12}. It is possible that these bacteroids might play an important part in the metabolism of oothecal uric acid.

There have been several reports that female Orthoptera consume a spermatophore which has been ejected after mating, and it may not be uncommon for a male to transfer materials of nutritional value in the accessory gland secretions^{6,13,14}.

Gordon¹⁵ has characterised cockroaches as being versatile omnivores capable of surviving on foods of low quality and limited quantity. Also, those nutrients most likely to be stored are those which may be of limited supply in the environment. Storage of urates (which are generally viewed to be end products of nitrogen metabolism and discarded by most higher uricotelic organisms) as a nitrogen resource by organisms adapted to utilise them in conditions of dietary stress has obvious advantages¹².

We suggest that male *B. germanica* urate secretions (Fig. 1) may represent paternal investment of a nitrogen resource from which the female and her progeny might benefit. The evidence presented that urates secreted during mating are incorporated into ootheca at various levels related to the dietary nitrogen status of the parents, and the discovery that uric acid is present in newly formed oothecae emphasise the importance of this material to this group of insects. However, little is known about catabolism of uric acid. Studies using ^{14}C -labelled purine intermediates reflect only pathways involving the carbon atoms and do not necessarily reflect the path of nitrogen contained in

Table 2 Distribution of radioactivity in whole oothecae obtained from female *B. germanica* which had been paired with labelled males for 38 days

Diet	Soluble fraction		Precipitated fraction		Oothecal cases	Total
	d.p.m.	Uric acid*	d.p.m.			
5% Protein	9,470 $\pm$ 2,751	58%	88 $\pm$ 18		163 $\pm$ 60	9,721 $\pm$ 2,790
25% Protein	727 $\pm$ 314	88%	15 $\pm$ 3		22 $\pm$ 8	760 $\pm$ 323

Frozen oothecal extracts obtained from the experiment described in Table 1 were heated, centrifuged and the supernatant (soluble fraction) counted in PCS/xylene solution. The precipitated pellet was resuspended and washed several times before suspension in the counting solution. The oothecal cases were extracted several times in heated buffer and ground to a very fine powder before placement in counting solution. The per cent uric acid content was obtained from pooled samples of the soluble fractions (5% protein and 25% casein protein) after purification by TLC (1-mm cellulose plates developed in n -butanol/methanol/ H_2O /60:20:20:1) and scintillation counting.

* Uric acid present in the urate spherules excreted by the males is partially potassium urate, hence we refer to it as urate. However, because nothing is known about its form in the ootheca, we refer to it as uric acid.

urates. Therefore, investigations designed to elucidate these relationships further might prove useful.

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Chronic blockade of dopamine receptors by antischizophrenic drugs enhances GABA binding in substantia nigra

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Antischizophrenic drugs exert their primary effects through blockade of dopamine (DA) receptors in the central nervous system (CNS)^{1–3}. However, because of intricate interconnections between neural pathways, alteration of DA function in the CNS can lead to changes in the function of other neurotransmitters. In the basal ganglia, the turnover and utilisation of several neurotransmitters, including γ -aminobutyric acid (GABA)^{4–6}, are indirectly influenced by nigrostriatal DA activity^{7–10}. However, although attention has been focused on changes in DA receptors^{11,12}, relatively little is known about changes in receptors for other transmitters after chronic antischizophrenic drug treatment. It is likely that changes occurring one or more synapses removed from the drugs' primary site of action may account for some of the time-dependent changes in the therapeutic and adverse clinical effects of antischizophrenic drugs^{13,14}. In the substantia nigra (SN), a reduction in GABAergic inhibitory tone and a possible increase in the release of excitatory transmitter such as substance P are effects which seem to accompany the acute blockade of striatal DA receptors¹⁵. Recent studies have shown that a chronic decrease in GABA function in the SN, through the destruction of striatonigral pathways^{16,17}, produces an increase in the number of GABA binding sites in the SN^{14,15}. Therefore, if a relative deficit of GABA transmission in the SN were to be maintained by chronic antischizophrenic drug treatment, an increase in nigral GABA binding might occur. To investigate this possibility, two classical prototype antischizophrenic drugs (chlorpromazine and haloperidol) were examined for their effects on specific GABA binding in the SN and striatum of rats. These effects were compared with those of clozapine, an antischizophrenic drug with an atypical biochemical and clinical profile^{4,18–23}. The data obtained, and reported here, support the hypothesis that chronic blockade of dopamine receptors by classical antischizophrenic drugs enhances GABA binding in the SN. This effect may be related to the extrapyramidal side effects associated with classical antischizophrenic drug treatment¹³.

Male Sprague–Dawley rats, weighing 200 g at the start of the experiments, were treated for 8 weeks with daily subcutaneous (s.c.) injections of haloperidol (0.7 mg per kg), chlorpromazine (20 mg per kg) or clozapine (20 mg per kg). At the end of the 8-week period, 5 days elapsed without drug administration before the rats were killed for measurement of ³H-GABA binding. Binding measurements were made in a crude synaptosomal-mitochondrial membrane preparation which had been frozen and thawed, treated with Triton X-100 (0.01% for 60 min at 37 °C) and washed extensively^{17,24,25}.

Table 1 shows the amount of specific ³H-GABA binding in the SN of drug-treated groups. When compared with values from control rats which received daily injections of vehicle, the values obtained from rats receiving either chronic chlorpromazine or haloperidol treatment were significantly elevated. In the same rats in which this increase in specific ³H-GABA binding was found in the SN, no change in specific ³H-GABA binding was obtained in the striatum (Table 1). In contrast, chronic clozapine treatment did not cause a significant change in the specific binding of ³H-GABA in the SN. No changes in specific GABA binding were observed in the SN after acute drug administration (1 h before killing), indicating the importance of chronic treatment for producing an increase in nigral GABA binding. The SNs from several chronically treated rats were pooled for binding studies using a range of GABA concentrations (10–90 nM). Scatchard analysis revealed that the increased GABA binding in the SN of chlorpromazine- and haloperidol-treated rats was due to an increase in B_{max} and not to a change in affinity (K_D) of the binding sites for GABA (see Table 1 legend).

Thus, the classical antischizophrenic drugs, chlorpromazine and haloperidol, although not acutely changing nigral binding sites for GABA, on repeated administration can cause an increase in the apparent number of these binding sites. As GABA binding in the striatum did not change, the increase observed in the SN does not represent a general response of brain GABA receptors to these drugs but probably reflects the juxtaposition of specific neural pathways. As in the case of lesions of striatonigral GABAergic projections^{16,17}, the increase in nigral GABA binding after chronic drug treatment may represent a compensation for a decrease in the synaptic activity of GABA. This possibility is consistent with the evidence that chronic treatment with haloperidol or chlorpromazine reduces the turnover rate of GABA in the SN⁴.

The lack of effect of chronic clozapine-treatment on GABA binding in the SN is probably related to the unique pharmacology of this compound. Clozapine does not cause catalepsy in animals and is devoid of Parkinson-like extrapyramidal side effects in man^{18,19}. There is evidence that the DA receptors affected by clozapine may be functionally distinct from those affected by haloperidol and chlorpromazine^{20–22}. In addition, clozapine's blockade of muscarinic cholinergic receptors²³ may interfere with this drug's ability to activate the striatonigral pathways required for the suppression of GABAergic transmission in the SN. Thus, acute clozapine treatment, unlike haloperidol or chlorpromazine, causes an increase in the turnover rate of GABA in the SN⁴.

To evaluate the possible significance of an alteration in nigral GABA receptors, the functional role of these receptors must be considered. Activation of GABA receptors in the SN can prevent the stimulation of tyrosine hydroxylase and DA synthesis which occurs in the striatum after an acute injection of haloperidol²⁶. Thus, an increase in SN GABA receptors after chronic haloperidol and chlorpromazine may contribute to the gradual decline in the ability of these drugs to enhance DA turnover^{22,27–30}. In addition, non-DA pathways originating in the SN zona reticulata are contacted by GABA terminals³¹ and probably contain GABA receptors^{32,33}. These latter pathways may mediate the DA-independent behavioural responses (contralateral rotation or stereotypies) evoked by the direct unilateral or bilateral activation of nigral GABA receptors in the rat^{34–38}. Stimulation of nigral GABA receptors can also

Table 1 ^3H -GABA specific binding (fmol per mg protein) in SN and neostriatum after chronic treatment with antischizophrenic drugs

Chronic treatment (mg per kg, s.c.) (8 weeks)	SN†	Striatum†
Control	382 ± 15	322 ± 13
Haloperidol (0.7)	501 ± 32* (131)	331 ± 18 (103)
Chlorpromazine (20)	521 ± 30* (136)	348 ± 13 (108)
Clozapine (20)	360 ± 19	338 ± 15 (105)

Values represent the means of 15 rats (3 separate experiments, 5 rats each) $\pm$ s.e.m. Rats were killed by decapitation and the SN and striatum dissected out as previously described¹⁵ and placed on dry ice. For each SN determination, both SNs (8 mg wet weight tissue) from one rat were combined; this yielded enough protein (300–400 μg in the final membrane preparation) to run triplicate samples for binding. Tissue was prepared according to the method of Enna and Snyder²⁴; as described by Toffano *et al.*²⁵, this preparation results in the removal of a membrane-bound protein inhibitor of high-affinity GABA binding. Binding was measured at 0 °C in a Na^+ -free medium in the presence of 30 nM ^3H -GABA; nonspecific binding was measured in the presence of 10^{-3} M nonradioactive GABA²⁴. The SN from groups of three or four rats were pooled to obtain values for Scatchard plots of high-affinity binding (10–90 nM ^3H -GABA)¹⁷. In these conditions, linear regression analysis of the data indicated that the K_D values of haloperidol (42 ± 5 nM) and chlorpromazine (40 ± 5 nM) rats were not significantly different from those of controls (36 ± 4 nM). Values of B_{max} (pmol per mg protein) were: control, 1.19 ± 0.06 ; haloperidol, $1.61 \pm 0.10^*$; chlorpromazine, $1.58 \pm 0.12^*$.

* Values significantly different from controls, $P < 0.05$.

† Numbers in parentheses refer to % of control.

reverse the catalepsy induced by haloperidol³⁶. These data suggest that GABA receptors in the SN, functioning downstream from the influence of DA neurones, may participate in the relay of neural output from the basal ganglia.

From this perspective, it is tempting to speculate that a modification of nigral GABA receptors, produced by classical antischizophrenic drugs, may be responsible for some of the behavioural consequences of their long-term administration. Thus, the development of tolerance to catalepsy²⁷ and the gradual increase in the ability of DA agonists to induce stereotyped behaviour^{39,40} in animals, as well as the disappearance of parkinsonian symptoms and the appearance of dyskinesias in humans¹³, are examples of processes which occur in response to chronic treatment with antischizophrenic drugs, and which may be brought about by 'supersensitive' receptors, not only for DA, but also for GABA.

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Effects of 4-aminopyridine on normal and demyelinated mammalian nerve fibres

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We have previously demonstrated that a drug which prolongs action potentials can, like a reduction in temperature, overcome conduction failure in demyelinated nerve fibres¹. Although the particular substance then used, a scorpion venom, was not a suitable therapeutic agent, we suggested that other drugs, with similar but milder effects on the action potential, might be effective in the symptomatic treatment of multiple sclerosis². We now report some encouraging results obtained with 4-aminopyridine (4AP), a substance which blocks the voltage-dependent potassium currents in squid giant axons³. The use of a potassium-blocking agent to prolong action potentials may seem surprising because we previously found tetraethylammonium chloride (TEA), another potassium blocker, ineffective on normal rat myelinated fibres¹, and two recent voltage-clamp studies have confirmed that mammalian nodes have few, if any, potassium channels^{4,5}. On the other hand, 4AP strongly potentiates transmitter release from the unmyelinated terminals of rat motor nerves⁶, and the possibility arose that demyelinated axon membrane, which can conduct impulses continuously like an unmyelinated fibre⁷, might further resemble its unmyelinated terminals by responding to 4AP. In testing this hypothesis, we have found that both TEA and 4AP prolong action potentials of demyelinated and unmyelinated fibres, and both facilitate conduction in fibres blocked by demyelination. 4AP is effective at lower concentrations, and is the more promising for clinical use, as it has already been used with beneficial effects in the treatment of Eaton-Lambert syndrome⁸ and myasthenia gravis⁹.

The contrasting effects of 4AP on normal A and C fibres are most readily seen by comparing the effects on the A and C elevations of the same dorsal root compound action potential. To obtain the records in Fig. 1, a rat was anaesthetised with urethane, and after laminectomy a lumbar dorsal root was raised on stimulating and recording electrode pairs in a paraffin pool maintained at 37 °C. The compound action potentials evoked at low and high stimulus strengths were recorded monophasically from near the cut central end. At low stimulus strength, the 4AP had no effects on the main A-fibre spike (although at high gain the after potential became more negative); the C-fibre

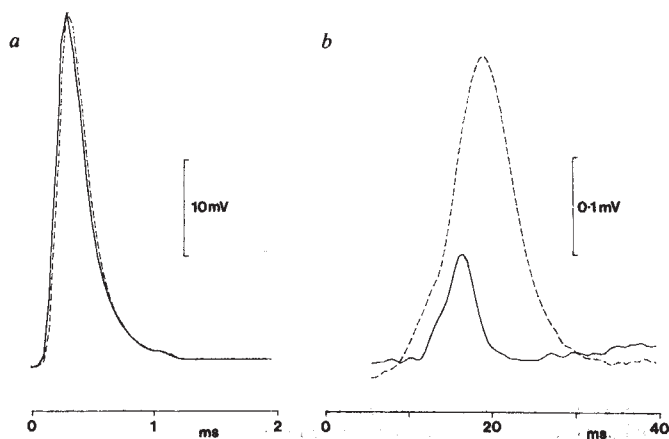


Fig. 1 Effect of 4AP on compound action potential in rat dorsal root, showing myelinated (*a*) and unmyelinated (*b*) fibre components separately. Each trace is the mean of eight, obtained with a digital signal averager. Solid lines, control; dashed lines, following application of 4 mM 4AP.

component, however, obtained by subtracting the low stimulus response from the high stimulus response, was increased in area severalfold. Of 17 dorsal roots treated with 5 mM 4AP, the increase in peak height of the C component ranged from 4% to 550%, with a median of 127%.

To determine whether the change in the C-fibre compound action potential was due simply to a prolongation or to some other change in the individual C-fibre action potentials, and to determine the effect on demyelinated axons, it was necessary to record from single fibres. Figure 2 shows typical examples of the effects of 4AP on the action potentials of single myelinated (*a*), unmyelinated (*b*) and demyelinated (*c*) nerve fibres, also recorded from rat spinal roots. In *b*, the single fibre was stimulated and recorded as in Fig. 1, and judged to be unmyelinated from its conduction velocity of 0.43 m s^{-1} at 30°C . It was functionally isolated from the myelinated fibres in a dorsal root filament by latency, and from the other unmyelinated fibres by its threshold. In *a* and *c* the action potentials of single fibres in sacral ventral roots were isolated by adjusting the position and intensity of a stimulus to the tail. In *c* the demyelination was caused by intrathecal injection of diphtheria toxin 12 d previously⁷. In each case digital averaging was used to improve the signal-to-noise ratio, and in *a* and *c* the averager sweep was triggered by the spike as described previously⁷. As with the compound dorsal root A spike, the single ventral root myelinated fibre action potential was unchanged by 5 mM 4AP. The single unmyelinated fibre action potential, on the other hand, was prolonged and increased slightly in amplitude. The demyelinated fibre had an action potential of irregular shape, as is usual, and this was also prolonged.

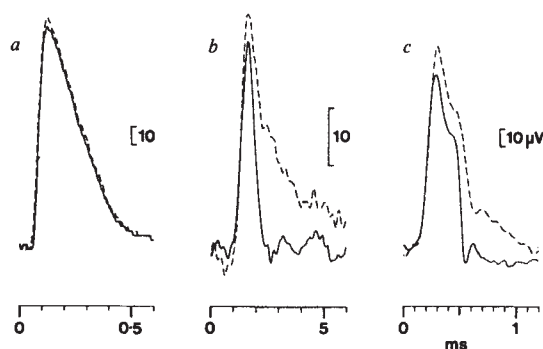


Fig. 2 Effects of 4AP on single fibre action potentials. *a*, Myelinated (37°C); *b*, unmyelinated (30°C); *c*, partially demyelinated fibre (37°C). Each trace is the mean of 64. Solid lines, controls; dashed lines, following application of 5 mM 4AP.

The mechanism of action potential prolongation is best revealed by records of membrane currents, which can be calculated by subtracting adjacent records of longitudinal current, made between closely spaced pairs of electrodes¹⁰. Membrane current starts in the outward direction as the approaching action potential depolarises the membrane, and in myelinated axons only becomes inward at the nodes, when they are excited. Figure 3 shows the effects of 4AP on the membrane currents at two consecutive nodes in a dorsal root fibre exposed to diphtheria toxin 6 d previously. Conduction in this fibre was saltatory, except for one demyelinated internode, over which conduction was continuous, at only 1.1 m s^{-1} (30°C). The abnormally large and slow currents in this region resembled those described previously for continuously conducting internodes¹¹. The node in Fig. 3*a* was unaffected by the toxin, judging from the small amount of outward current required to depolarise it to threshold, and the 4AP had little effect. The currents in Fig. 3*b* were recorded from the next 'node' 1 mm away, which was also the start of continuous conduction. As at succeeding electrode positions in the demyelinated internode, the membrane currents had three conspicuous phases: outward current during passive

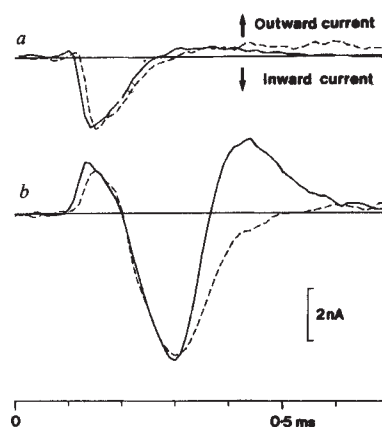


Fig. 3 Effects of 4AP on membrane currents of two consecutive nodes 1 mm apart in single dorsal root fibre exposed to diphtheria toxin. Each trace obtained by subtracting averaged longitudinal currents ($n = 64$) recorded $200 \mu\text{m}$ apart, so that points below the baseline denote inward current. Solid lines, controls; dashed lines, following application of 5 mM 4AP.

depolarisation, active inward current and a late outward current phase. This late phase of outward current, occurring at the time an active potassium current would be expected, was abolished by 4AP, although conduction along the internode persisted at 1.1 m s^{-1} .

We have observed the abolition of a late phase of outward current in two other continuously conducting internodes treated with 4AP. We have also observed a similar effect at individual nodes showing signs of paranodal demyelination after diphtheria toxin injection. Of 30 nodes examined as in Fig. 3, we have found a prolongation of net inward current only at those eight nodes which took much longer than normal to be excited. These results strongly suggest that the axon membrane exposed by demyelination has different excitability properties from those of normal nodes, with a much higher proportion of potassium channels. This interpretation must remain speculative without voltage-clamp data, but receives some support from a recent voltage-clamp study of single rat nodes: potassium currents, normally weak compared with amphibian nodes, were increased in rats made diabetic with alloxan⁴. Whatever the explanation, the extranodal membrane more closely resembles, in its response to 4AP, the membrane of unmyelinated fibres, than that of normal nodes.

If 4AP prolongs action potential at demyelinated, but not at normal nodes, it might be expected to aid conduction through a

diffuse demyelinating lesion, although it could not help transmission from the last normal node to the first affected one. We have tested the effects of 4AP on the blocking temperatures of 16 demyelinated single fibres, using the method previously described for scorpion venom¹. The blocking temperature was raised (that is, transmission was improved) in 12 and lowered in only two cases, although in four cases the responses did not follow a simple time course. The effects were not as striking as those of scorpion venom¹, which acts by inhibiting sodium inactivation, but in view of the limited prolongation of the action potential that could occur with a blocking agent for potassium channels they were encouraging. One reason for testing 4AP was that it has already been used clinically and can be tolerated in patients at a concentration sufficient to enhance transmitter release from unmyelinated motor nerve terminals^{8,9}. There are grounds for hoping that potassium channel blockers such as 4AP will also prove to be of value in the symptomatic treatment of multiple sclerosis and other demyelinating disease, through their specific action at those sites in myelinated fibres that most resemble their unmyelinated terminals.

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Abrogation of genetically controlled resistance of mice to *Treponema pallidum* by irradiation

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Infection with *Treponema pallidum*, the causative agent of human syphilis, gives rise to a complex immune response involving both humoral and cellular components. The exact nature of this response and how it relates to the disease process is a matter of considerable speculation^{1,2}. In recent years, studies have been directed towards defining the role of cell-mediated immunity (CMI) in syphilis². These have been conducted mainly *in vitro* because the general unavailability of inbred rabbits, the principal animals for experimental syphilis research, has limited the application of *in vivo* procedures. A prime deterrent to using mice for the study of syphilis has been their failure to exhibit pathology, even in the face of a persistent infection^{3,4}. We report here that on intradermal (i.d.) infection, transient primary lesions, characteristic of those seen in naturally acquired human syphilis, can be produced regularly in some strains of mice but not others, indicating a genetic basis for host susceptibility. Strains of mice which normally fail to develop lesions, do so after exposure to ionising radiation. Evidence is presented for a multiple role of the immune response during local infection.

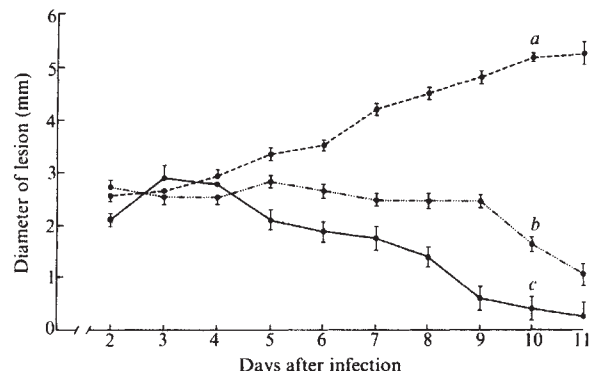


Fig. 1 Progression of lesion size in infected C3H/He mice. Each point represents the mean value $\pm$ s.e.m. of 15 normal mice (c), 10 irradiated mice reconstituted with non-immune cells as described for Table 2 (b), and 15 irradiated mice (a). The kinetics of lesion development seen in irradiated BALB/c and Swiss mice were the same as shown for C3H/He mice (a). Inoculation procedures consisted of i.d. injection into an area of the backs of anaesthetised mice from which hair had been removed by clipping and the application of a depilatory cream. Each animal received 0.01 ml of a suspension of *T. pallidum*, 6×10^8 per ml, derived from an orchitic rabbit preparation as previously described¹², and an equal volume of the same suspension rendered non-infectious by aerobic storage for 4 d at 4°C. All mice were maintained at 16–18°C, temperatures which are known to promote cutaneous pathology in other *T. pallidum*-infected animals⁸. Inocula of $>10^4$ organisms were required to elicit a cutaneous lesion.

Mice infected i.d. and housed at 16–18°C develop prominent lesions at the site of inoculation within 48 h, appearing as well demarcated, indurated papules which increase in size until day 4 of infection, and then gradually recede (Fig. 1). Depending on mouse strain, 40–60% of lesions become necrotic and form an overlying crust of dry inflammatory exudate from days 4 to 9 of infection. Histologically, these lesions are characteristic of the pathologic changes of primary syphilis. Beginning on day 2 of infection, there is an accumulation of mononuclear cells consisting of macrophages, lymphocytes and, subsequently, plasma cells throughout the epidermis and dermal collagen and fat. About one half of the lesions progress to ulceration or display a marked thinning of the epidermis beneath an accumulation of dense nuclear debris and inflammatory exudate, persisting until

Table 1 Frequency of lesions in *T. pallidum*-infected normal and irradiated mice

Strain	H-2 haplotype	No. mice with lesions/ no. tested (%)	
		Non-irradiated	Irradiated
Swiss	—	3/40 (7.5)	40/40 (100)
C3H/He	k	40/50 (80)	43/43 (100)
DBA/2	d	14/35 (40)	NT
C57BL/6	b	20/54 (37)	NT
BALB/c	d	8/40 (20)	35/35 (100)
AKR	k	2/10 (20)	NT
(C3H/He $\times$ BALB/c) F_1	kd	10/14 (71)	NT

Lesions were scored at day 4 of infection, the time of maximum frequency in non-irradiated mice. By day 2 of infection, all irradiated mice developed lesions which persisted until death. Using a bacterial counting chamber, the concentration of treponemes in undiluted, wet-mounted preparations of serous material taken from 8-d lesions in irradiated C3H/He mice was $3.15 \pm 0.22 \times 10^9$ per ml (mean of 5 mice $\pm$ s.e.m.); similar numbers of treponemes were found in material from lesions of irradiated BALB/c and Swiss mice. By χ^2 analysis, lesion frequencies in C3H/He mice differed significantly ($P < 0.005$) from all other strains except the (C3H/He $\times$ BALB/c) F_1 group. Frequencies in BALB/c and AKR mice were the same; differences between BALB/c and DBA/2 mice were not highly significant ($P = 0.05$). NT, not tested.

days 7 to 10 of infection. Both gross and microscopic characteristics are similar for all strains of mice tested. Sites of control inoculations with non-infectious organisms exhibit only transient, colourless blemishes during the first 24–48 h, and never develop crusted surfaces. Histological changes are minimal, consisting of occasional mild suppurative inflammation which resolves within 24–48 h of inoculation.

As shown in Table 1, there is marked variation in the susceptibility of mice to lesion development which is least in outbred animals, a significant point as most published studies of murine syphilis have involved such animals^{3,5}. Inbred strains display a spectrum of susceptibility with C3H/He mice developing lesions most consistently. The high lesion frequency seen in (C3H/He × BALB/c)F₁ mice may suggest that the factor(s) responsible for the clinical manifestation of infection are under genetic control, although more detailed studies along these lines will be required to define the exact nature of this relationship. Susceptibility seems not to be influenced by H-2 haplotype, a situation also applying for certain other bacterial infections of mice^{6,7}. Consistent with the phenomenon of chancre-immunity that occurs in human syphilis and experimental *T. pallidum* infections of other animals⁸, mice which develop primary lesions become completely resistant to i.d. treponemal challenge by day 20 of infection.

The importance of an intact immune system in the outcome of local infection was dramatically illustrated by the use of radiation-induced immunosuppression. Mice were exposed to lethal doses of total body irradiation (TBI) from a ¹³⁷Ce source (137 rad per min), 850–1,050 rad depending on mouse strain⁹. Mice, infected 24 h after irradiation, developed progressively enlarging soft colourless lesions with rounded surfaces that persisted until the time of death 10–14 d post-irradiation. Lesions in all irradiated mice, including those of 'resistant' strains, contained extraordinarily large numbers of viable treponemes and never

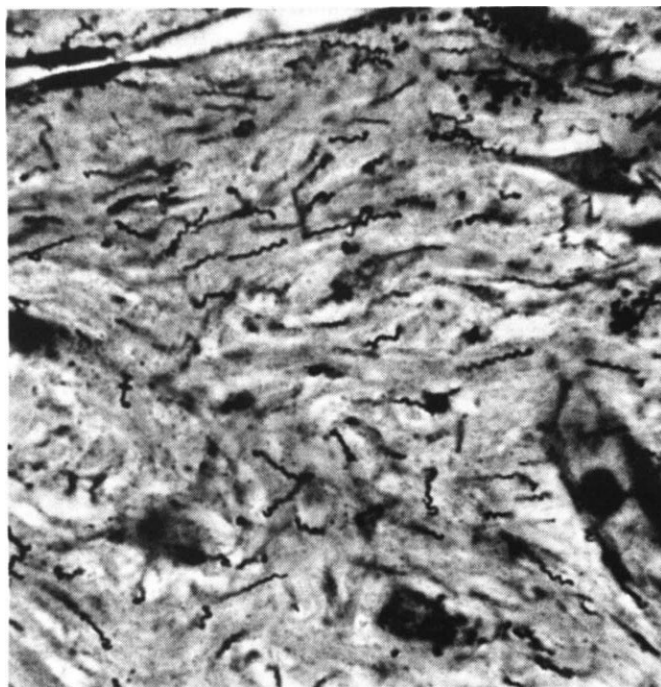


Fig. 2 Warthin–Starry-stained section of an 8-d lesion from an infected irradiated C3H/He mouse (×930). The pronounced accumulation of spirochetes in the absence of appreciable pathology is typical of lesions examined in all strains of irradiated mice (5 mice per strain). [Warthin–Starry-stained sections of lesions from non-irradiated mice reveal a gradual reduction in the number of spirochetes until day 5 of infection, after which no organisms are identifiable.]

Table 2 Effect of irradiation and immune reconstitution on cutaneous lesions in C3H/He mice

Recipient	TBI (950r)	Spleen cell donor	Challenge outcome	
			No. of mice with lesions/ no. tested	Type of lesion
Non-immune	–	–	40/50	Healed
	+	–	40/40	Unhealed
	+	Non-immune	10/10	Healed
	–	Immune	0/10	None
	+	Immune	0/10	None
Immune	–	–	0/13	None
	+	–	9/9	Unhealed

Non-immune mice were age-matched uninfected animals. Immune mice were animals infected i.d. 23–28 d previously which developed primary lesions. As measured using an indirect fluorescent antibody procedure¹¹, the mean anti-treponemal antibody titre of immune mice was 32 (the reciprocal of the highest serum dilution giving positive fluorescence). Healed and unhealed lesions are as described in the text for non-irradiated and irradiated mice respectively. Syngeneic spleens were teased through a 60-mesh stainless steel screen. The cells were suspended and washed twice in Hanks' balanced salt solution. Cell viability was >95% as determined by Trypan blue exclusion. Animals were reconstituted with either 8×10^7 nucleated non-immune cells given intraperitoneally, or 4×10^7 nucleated immune cells given intravenously.

exhibited the ulcerative appearance frequently seen in immunocompetent mice (Table 1). Highly motile organisms were readily recoverable from all lesions sampled, particularly between days 6 and 11 of infection, a time when treponemes were virtually absent in lesions of non-irradiated mice. Similarly, Warthin–Starry-stained paraffin sections of lesions revealed an intense accumulation of treponemes throughout their entire area (Fig. 2).

Immunological involvement was further demonstrated by reconstituting C3H/He mice with syngeneic spleen cells within 2 h of irradiation, a procedure which not only altered the course of lesion development (Table 2, Fig. 1), but also prevented radiation death. Recipients of non-immune spleen cells, when infected 2–3 d later, developed indurated crusted lesions, similar to those seen in infected non-irradiated animals, and healing within 11 d. However, irradiated mice infected 24 h after reconstitution (data not shown) developed lesions which, like those of irradiated unreconstituted mice, progressed until day 7 of infection, except that they then became indurated and ulcerated, requiring an additional 10–12 d to heal. In these conditions, it seems that treponemes became firmly established in cutaneous tissues before immune function was restored, resulting in a biphasic lesion morphology. Collectively, these results indicate that potentiation of treponemal infection in irradiated mice is primarily the result of abrogating the immune response. As shown in Table 2, recipients of immune spleen cells, with or without preceding irradiation, are protected against any type of lesion development after i.d. challenge. However, irradiation of immune mice reestablishes their susceptibility and, when challenged, they develop large papular lesions, rich in motile treponemes, and identical to those produced in non-immune irradiated mice. Note that irradiation had no detectable effect on existing anti-treponemal antibody levels in immune mice when tested 10 d post-irradiation (Table 2).

This study points to two distinct roles for CMI during local *T. pallidum* infections. First, it undoubtedly is responsible for the characteristic pathogenesis of primary lesions as has been suggested from previous studies of treponemal infections in rabbits immunosuppressed with cyclophosphamide or cortisone^{8,10}. By combining irradiation of the murine host with immune reconstitution, we have obtained direct evidence for the immune-mediated basis of these lesions. Second, the cellular component of the anti-treponemal immune response seems to

be required for both the resolution of local infection and protection against subsequent i.d. challenge; its absence permits treponemes to thrive at the site of infection with only minimal tissue damage. We consider this model to have much potential application to the study of syphilis immunobiology since it should enable the identification of *T. pallidum*-specific effector cells and permit their functional activity to be defined *in vivo*. It may also serve as a new tool for evaluating both the virulence of different strains of *T. pallidum* and the immunoprophylactic value of potential non-infectious syphilis vaccines, including purified treponemal antigens. Finally, the model lends itself to further studies of host genetic factors in disease susceptibility.

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Requirement for monocytes in the spontaneous cytotoxic effects of human lymphocytes against non-lymphoid target cells

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Human lymphocytes have been shown to lyse *in vitro* a large variety of target cells derived from tumour as well as normal tissues^{1,2}. The effector cells involved in this spontaneous cell-mediated cytotoxicity (SCMC) have generally been characterised as lymphocytes which lack surface membrane immunoglobulin (sIg) and lack or express only low-affinity receptors for sheep red blood cells (SRBC), but possess receptors for the Fc portion of IgG (refs 3–7). We have previously shown that monocytes may be involved in SCMC, as strong depletions of adherent cells always resulted in a reduction in SCMC⁸. In the present study, experiments were undertaken to determine the role of monocytes in SCMC. Evidence is presented that monocytes have a helper function in the lymphocyte-mediated SCMC against target cells growing in monolayer cultures, whereas effector cells in SCMC against lymphoid target cells growing in suspension cultures were lysed by lymphocytes in the absence of monocytes.

SCMC was tested in a 16-h ⁵¹Cr-release assay against target cells from T-24 bladder carcinoma, NKI-1 and NKI-10 melanoma, and EK embryonic kidney cell lines, growing in monolayers, and in a 4-h ⁵¹Cr-release assay on lymphoid target cells from K562 (myeloid leukaemia) and Molt-4 (T-cell leukaemia) growing in suspension cultures. All cultures and tests were carried out in Dulbecco's modified Eagle's minimal essential medium (DMEM) supplemented with antibiotics and 10% fetal calf serum (FCS). Ficoll/Hypaque-isolated mononuclear leukocytes from peripheral blood, very pure lymphocyte frac-

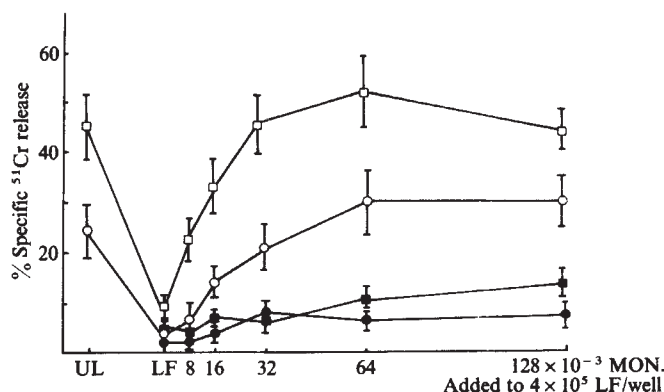


Fig. 1 Restoration of SCMC with autologous monocytes (mean $\pm$ s.e.m. of four experiments). 10^6 Monocytes were incubated for 3 h at 37°C in 1 ml culture medium containing 300 μ g carrageenan under constant shaking. Before testing, the monocytes were washed three times by centrifugation. □, T-24; ○, NKI-1; ■, LF tested on T-24 after addition of carrageenan-treated autologous monocytes; ●, LF tested on NKI-1 after addition of carrageenan-treated autologous monocytes.

tions (LF) and highly enriched monocyte fractions (MF) were used as effector cells. The LF and MF were obtained by separating the Ficoll/Hypaque-isolated mononuclear leukocytes according to size by velocity sedimentation at unit gravity in a LACS-II apparatus⁹. Both fractions are characterised in Table 1.

Compared with the corresponding unfractionated mononuclear leukocytes (UL), the LF and MF of 14 different healthy donors showed markedly reduced SCMC against the monolayer targets. In contrast, LF cells were nearly as effective as the corresponding UL in SCMC against K562 and Molt-4 target cells growing in suspension cultures, whereas the MF cells were insignificantly or weakly cytotoxic for these targets.

To investigate whether the loss of SCMC of the LF against monolayer targets could be attributed to the monocyte depletions, reconstitution experiments were carried out with autologous MF cells (Fig. 1). Addition of approximately 2% monocytes to LF cells resulted in significantly increased SCMC, whereas 8–15% monocytes were sufficient to restore the SCMC to the level achieved with the corresponding UL. Optimal induction of SCMC was observed after addition of 15–30%

Table 1 Characterisation of leukocyte fractions

	UL	LF	MF
		20–36 mm	50–62 mm
		h ⁻¹	h ⁻¹
Sedimentation velocity	—		
% Monocytes (esterase)	23 $\pm$ 4	0.2	83 $\pm$ 4
% Monocytes (phagocytosis)	20 $\pm$ 5	<0.1	79 $\pm$ 5
% Monocytes (electronic sizing)	22 $\pm$ 4	—	81 $\pm$ 4
% Lymphocytes	77 $\pm$ 5	99.8	16 $\pm$ 4
% Lymphocytes forming SRBC rosettes	51 $\pm$ 6	56 $\pm$ 6	9 $\pm$ 3
% Lymphocytes with Fc receptors	15 $\pm$ 3	17 $\pm$ 4	10 $\pm$ 3
% Lymphocytes with C ₃ receptors	18 $\pm$ 4	20 $\pm$ 3	5 $\pm$ 2
% Lymphocytes with sIg	6 $\pm$ 1	7 $\pm$ 2	3 $\pm$ 1
Granulocytes (basophils)	<0.5	0	1

Characterisation of the UL, LF and MF (mean $\pm$ s.d. of five different donors). The different criteria used to characterise the UL, LF and MF were described previously¹⁰. In all instances, at least 300 cells were counted. For estimating the monocyte contamination of the LF, up to 5,000 cells were screened. Of the lymphocytes present in the cell sample, 60% were recovered in the LF, whereas approximately 40% of the monocytes were obtained in the MF.

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Table 2 SCMC of unfractionated lymphocytes (UL), lymphocyte fraction (LF) and monocyte fraction (MF)

No. of effector cells added	T-24			NKI-1			NKI-10			EK			K562			Molt-4		
	UL	LF	MF	UL	LF	MF	UL	LF	MF	UL	LF	MF	UL	LF	MF	UL	LF	MF
4×10^5	48±7	8±3	9±3	34±6	6±3	8±3	20±4	2±1	5±2	55±9	10±2	13±3						
2×10^5	35±5	6±3	6±2	24±5	4±2	5±2	14±4	2±1	3±2	41±7	7±2	9±2	55±5	48±7	16±3	39±4	35±5	9±2
1×10^5	23±4	4±2	3±1	17±4	2±1	4±1	9±3	0	1±1	30±5	3±1	5±2	38±5	31±4	9±2	28±5	26±4	7±2
5×10^4													31±4	25±4	4±1	20±4	19±3	5±2

SCMC (mean % of specific ^{51}Cr release $\pm$ s.e.m.) of UL and corresponding LF and MF from 14 different healthy donors was tested on T-24, NKI-1 and MKI-10 target cells. Six different donors were tested on EK, K562 and Molt-4 target cells. The ^{51}Cr -release assays on monolayer targets (T-24, NKI-1, NKI-10 and EK) were carried out in Microtest II plates (Falcon, no. 3040 F). Confluent monolayers of target cells were trypsinised. Target cells 5,000 per well were plated in 0.2 ml culture medium and incubated overnight at 37°C and 5% CO_2 , and then 4 μCi ^{51}Cr was added to each well. After 4–6 h incubation, the target cells were washed three times and 0.2 ml of effector cells in culture medium was added in the concentrations indicated. Effector and target cells were incubated for 16 h after which 100 μl supernatant was collected and counted. The ^{51}Cr -release assays on K562 and Molt-4 target cells (growing in suspension cultures) were carried out in U-bottomed Linbro plates (no. 76-213-05). In these, 10^4 ^{51}Cr -labelled target cells (100 μl) were mixed with the appropriate number of effector cells (in 100 μl), centrifuged for 2 min at 150g and incubated for 4 h at 37°C and 5% CO_2 . At the end of the incubation 100 μl supernatant was collected and counted. The specific ^{51}Cr release was calculated by the following formula: % cytotoxicity = (test ^{51}Cr release – spontaneous ^{51}Cr release)/(total amount of ^{51}Cr incorporated into the targets – spontaneous ^{51}Cr release). The average spontaneous ^{51}Cr release $\pm$ s.d. was: T-24 = 18 $\pm$ 3; NKI-1 = 15 $\pm$ 3; NKI-10 = 13 $\pm$ 3; EK = 20 $\pm$ 4; K562 = 9 $\pm$ 2; Molt-4 = 12 $\pm$ 2.

monocytes. MF cells in the absence of LF cells were not cytotoxic. Intact, paraformaldehyde-killed monocytes were ineffective in inducing SCMC (not shown).

As the MF used as monocyte source for our reconstitution experiments contained 15–20% lymphocytes, it was possible that the induction of SCMC was mediated by these non-cytotoxic lymphocytes present in the MF. To evaluate this possibility, the MF cells were preincubated with carrageenan, which selectively kills monocytes¹¹. The carrageenan-treated monocytes caused no induction of SCMC after addition to the LF (Fig. 1), suggesting that the helper effects were mediated by monocytes and not by the contaminating lymphocytes in the MF. Lymphocytes were not affected by the carrageenan treatment, because LF cells preincubated with carrageenan showed good responses to mitogens after addition of autologous monocytes (J.E.d.V. and L. Holley, unpublished findings). Having demonstrated that SCMC on target cells growing in monolayer cultures required the presence of viable monocytes, we next tried to determine whether soluble factors produced by cultured monocytes could induce SCMC by LF cells. Figure 2 shows that supernatants of allogeneic monocyte cultures added to LF cells induced significant SCMC, although this was always weaker than the SCMC achieved with viable autologous monocytes and never exceeded 60% of the SCMC obtained with the corresponding UL. Monocytes or monocyte culture supernatants (MCS) could not be replaced by 2-mercaptoethanol (2-ME), which also had no supplementary effect on the restorative effects of MCS.

The effector cells in SCMC against non-lymphoid target cells growing in monolayers have previously been characterised as non-SRBC-rosette forming, sIg-negative, FcR⁺ lymphocytes^{7,13}. FcR⁺ lymphocytes were also found to be the actual effector cells in monocyte-induced SCMC by LF cells, because addition of autologous monocytes to LF cells depleted in FcR⁺ lymphocytes resulted in insignificant induction of SCMC (results not shown).

These results suggest that viable monocytes or soluble monocyte products are required for FcR⁺-lymphocyte-mediated SCMC against non-lymphoid target cells growing in monolayer cultures. Reconstitution with very low monocyte concentrations was sufficient to induce significant SCMC. This may explain why conventional monocyte depletion procedures, because they generally result in incomplete monocyte removal, failed to demonstrate the requirement of monocytes in SCMC measured in 16-h ^{51}Cr -release assays.

The finding that LF cells were completely able to kill K562 and Molt-4 cells in the absence of monocytes (Table 2) suggests that distinct types of effector cell are involved in the killing of target cells growing in monolayers and target cells of lymphoid

origin growing in suspension cultures (the former requiring monocyte help, the latter independent of it). Our findings are consistent with the results of Timonen and Saksela¹², who demonstrated that depletion of lymphocytes which were cytotoxic for fetal fibroblasts growing in monolayer cultures did not result in reduction of SCMC against lymphoma (Raji) and leukaemia (Molt-4, K562) cell lines. This indicates that different effector cell populations may be involved in SCMC against monolayer targets and lymphoid target cells growing in suspension cultures. Such a possibility is further supported by the finding that, in contrast to the effector cells active in SCMC against monolayer targets^{7,13}, the effectors in SCMC against K562 may possess low-affinity receptors for SRBC⁶. However, the extent to which the monocyte-independent SCMC against K562 and Molt-4 is related to the lymphoid origin and sensitivity of these cells remains unknown.

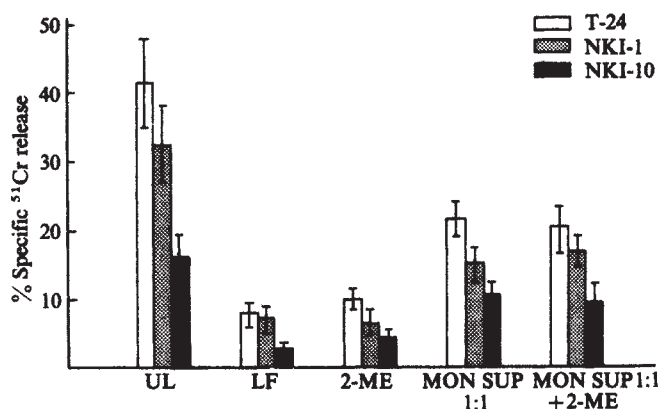


Fig. 2 Restoration of SCMC of LF cells by allogeneic monocyte culture supernatant (MCS). MCS was obtained from cultured MF cells. 4×10^5 MF cells in 0.2 ml DMEM + 10% FCS were cultured per well in Microtest II plates (Falcon, no. 3040 F). After overnight incubation (37°C, 5% CO_2) the wells were vigorously washed to remove non-adherent cells. Fresh culture medium (0.25 ml) was added and the plates were incubated for another 72 h (in other experiments 24–48 h) (37°C, 5% CO_2). The supernatants of these confluent monocyte monolayers (>96% esterase positive) were collected, centrifuged for 10 min at 2,000g, filter sterilised and stored at -80°C until testing. 2-ME = 2-mercaptoethanol (5×10^{-5} M) added to LF cells from three different donors (mean $\pm$ s.e.m.); MON SUP 1:1 = MCS 1:1 diluted in culture medium added to LF cells from three different donors (mean $\pm$ s.e.m.); MON SUP 1:1 + 2-ME = MCS 1:1 diluted in culture medium and 2-ME (5×10^{-5} M) added to LF cells (mean $\pm$ s.e.m.).

As LF cells were found to be cytotoxic for Chang liver cells in an antibody-dependent cellular cytotoxicity (ADCC) system¹³, we considered the possibility that the monocyte requirement for SCMC against monolayer targets could have been mediated by antibodies synthesised in the presence of, or shed by, monocytes. It has been reported that these types of antibodies may have a role in SCMC and that SCMC actually is a form of ADCC¹⁴⁻¹⁷. However, we found that goat F(ab')₂ fragments of purified anti-human Fab antibodies did not significantly inhibit SCMC, suggesting that antibodies are not involved in SCMC and that SCMC and ADCC must be attributed to distinct cytotoxic events¹³. It seems more likely that the induction of SCMC by monocytes is mediated by non-Ig soluble factors, as it

has been demonstrated that SCMC is enhanced by purified interferon¹⁸ and by interferon synthesised during co-cultivation of lymphocytes with tumour cells^{19,20}. Therefore, the possibility must be considered that in our system the induction of SCMC is also mediated by interferon produced either directly by monocytes or indirectly in cooperation with LF cells^{21,22}. These regulatory functions of monocytes in SCMC will be further investigated.

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T-lymphocyte differentiation is accompanied by increase in sialic acid content of Thy-1 antigen

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Lymphocyte differentiation is accompanied by changes in the expression of various glycoproteins at the cell surface¹⁻⁴. Whether changes in the structures of individual glycoproteins, as opposed to changes in their expression, also occur during this differentiation is not known. To answer this question, we have studied the structures of the Thy-1 glycoprotein isolated from mouse thymic (immature) and peripheral (mature) T lymphocytes. This glycoprotein was chosen for study because there is already evidence that the Thy-1 glycoprotein can carry different carbohydrate structures on the same polypeptide backbone⁵ and because it is also known that peripheral T lymphocytes have more sialic acid and more sialyl-transferase activity than do thymocytes⁶. Barclay *et al.*⁵ indeed showed that there were differences between the carbohydrate compositions of the Thy-1 antigen isolated from rat brain and thymus. We believe this to be the only clear example of tissue-specific differences in carbohydrate content of a given glycoprotein. In contrast to Barclay *et al.*, we have studied cells of a common differentiation pathway. Here we show, using a two-dimensional electrophoresis technique⁷, that differentiation can involve the modification of carbohydrate structures of individual glycoproteins, as Thy-1 molecules on peripheral T cells carry more sialic acid than do thymic Thy-1 molecules. We also show that there is a charge difference (which does not involve sialic acid) between the two allelic forms, Thy-1.1 and Thy-1.2, of the antigen.

Thymocytes or mesenteric lymph node cells were prepared from AKR and BALB/c mice, which carry the *Thy-1.1* and *Thy-1.2* alleles, respectively. The cells were radio-iodinated and lysed in NP-40 detergent⁸. The Thy-1 antigen was precipitated from the lysates⁹ using a rabbit antiserum prepared against a highly purified mouse thymus lymphocyte antigen (MTLA)

which is identical with, or at least very similar to, the Thy-1 antigen present on thymocyte membranes^{10,11}.

Figure 1 shows that anti-MTLA specifically precipitates, from BALB/c thymocytes, a series of basic spots (*pI* above *pH* 7.5) differing in charge, with each spot showing apparent molecular weight (MW) heterogeneity in the range 25,000-30,000. Very similar spots are precipitated by conventional anti-Thy-1.2 alloantisera, or monoclonal rat anti-mouse Thy-1 reagents¹². These results are in agreement with previous findings that the Thy-1 molecule is a glycoprotein of apparent MW about 25,000 (refs 5, 13).

Thy-1 antigens precipitated by anti-MTLA from thymocytes and lymph node cells of AKR and BALB/c mice are compared in Fig. 2. In both strains, the lymph node Thy-1 antigens are clearly more acidic than the thymus antigens. In the case of the lymph node, the spots are less heterogeneous in apparent MW, and the most acidic lymph node Thy-1 spots have a higher apparent MW than the least acidic. Similar results were obtained from splenic Thy-1 molecules.

The charge difference between thymocyte and peripheral cell Thy-1 molecules is entirely due to the presence of extra sialic acid in the peripheral lymphocyte antigens. As shown in Fig. 3, for AKR cells, the charge difference between thymocyte and lymph node cell Thy-1 molecules is abolished if the cells are treated with neuraminidase. The same is true for BALB/c cells (not shown). The thymocyte antigens do carry some sialic acid, as they become even more basic after neuraminidase treatment, but the amount of sialic acid on the Thy-1 of peripheral cells is clearly greater than that of thymocytes.

Almost all Thy-1 molecules on peripheral cells are more heavily sialylated than thymocyte Thy-1 (Figs 2, 3). The increase in sialylation is therefore probably not responsible for the antigenic difference reported to exist between the Thy-1 on thymocytes and a subpopulation (about 50%) of splenic T cells¹⁴. Moreover, neuraminidase-treated Thy-1 was readily precipitated by anti-MTLA, or a monoclonal anti-Thy-1 reagent (not shown).

Figure 2 also shows that AKR and BALB/c Thy-1 molecules differ in charge, with the AKR (Thy-1.1) spots being more basic than the corresponding BALB/c (Thy-1.2) spots. This inter-strain difference can also be seen in gels made from total lysates of iodinated thymocytes or peripheral cells, and is therefore not an artefact of the precipitation procedure. Also, Thy-1 molecules from AKR/Cum mice, which carry the Thy-1.2 specificity on an AKR background, behave like BALB/c Thy-1.2 mole-

Fig. 1 Immunoprecipitation and two-dimensional gel analyses of the Thy-1.2 antigen. 50×10^6 viable BALB/c thymocytes were incubated in 1 ml of phosphate-buffered saline (PBS), 20 mM D-glucose, for 40 min at 20 °C, with 10 μ g of lactoperoxidase (Sigma), 2 U of glucose oxidase (Sigma) and 1 mCi 125 I (NEN). After three washes in cold PBS, the labelled cells were lysed in 100 μ l of PBS containing 0.5% NP-40 (v/v) and 1 mM phenylmethylsulphonyl fluoride. After a low speed centrifugation to remove nuclei, the lysate was desalted on Sephadex G-25 and the labelled macromolecular material collected in the void volume. After centrifugation at 100,000g for 30 min, immunoprecipitation was carried out on aliquots of lysate corresponding to 10×10^6 cells⁹. *a*, Immunoprecipitation with 5 μ l anti-mouse T-lymphocyte antigen (MTLA) antiserum¹⁰; *b*, immunoprecipitation with 15 μ l of an anti-Thy-1.2 alloantiserum (AKR anti-C3H, absorbed on AKR thymocytes) for 30 min at 20 °C, then overnight at 4 °C, followed by 25 μ l of a rabbit anti-mouse Ig; *c*, immunoprecipitation with 15 μ l of a rat monoclonal anti-Thy-1.2 antibody; 50 μ l of a sheep anti-rat whole serum was added as a second antibody. Immunoprecipitated radioactivity was then extracted in 50 μ l of O'Farrell's isoelectric focusing buffer and run in non-equilibrium pH gel electrophoresis (NEPHGE)⁷ conditions. 10% polyacrylamide slab gels were used for the second dimension. Dried slab gels were exposed for 15–30 days to X-Omat R films (Kodak) with an intensifying screen. The basic end is at the left and the acidic end at the right of each panel, where the sample was loaded.

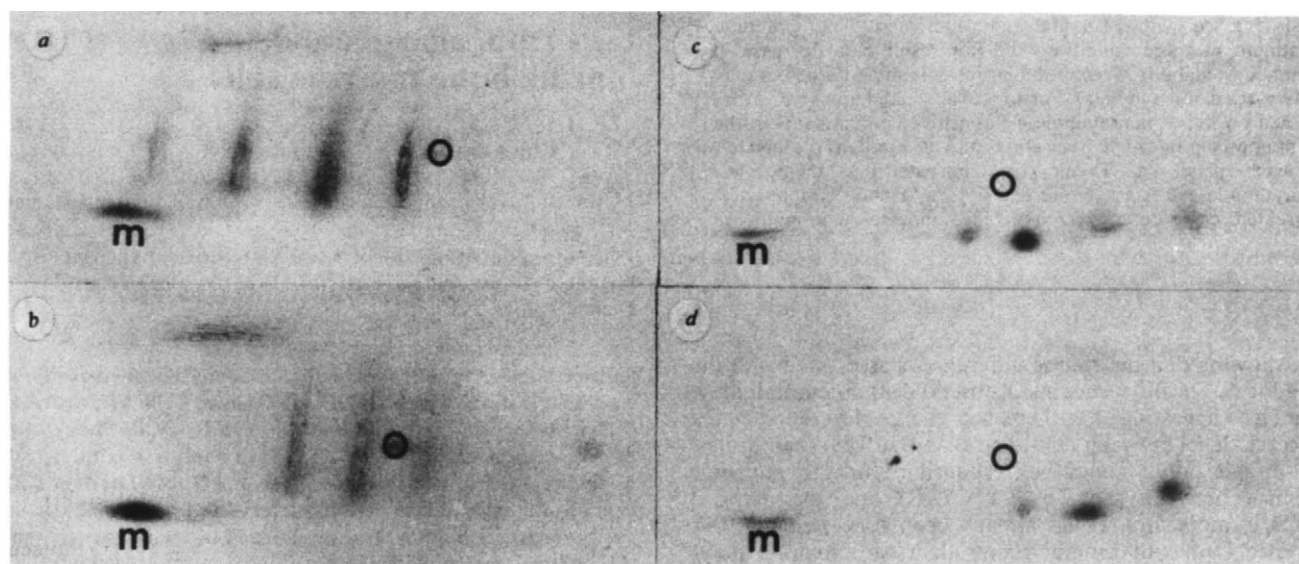
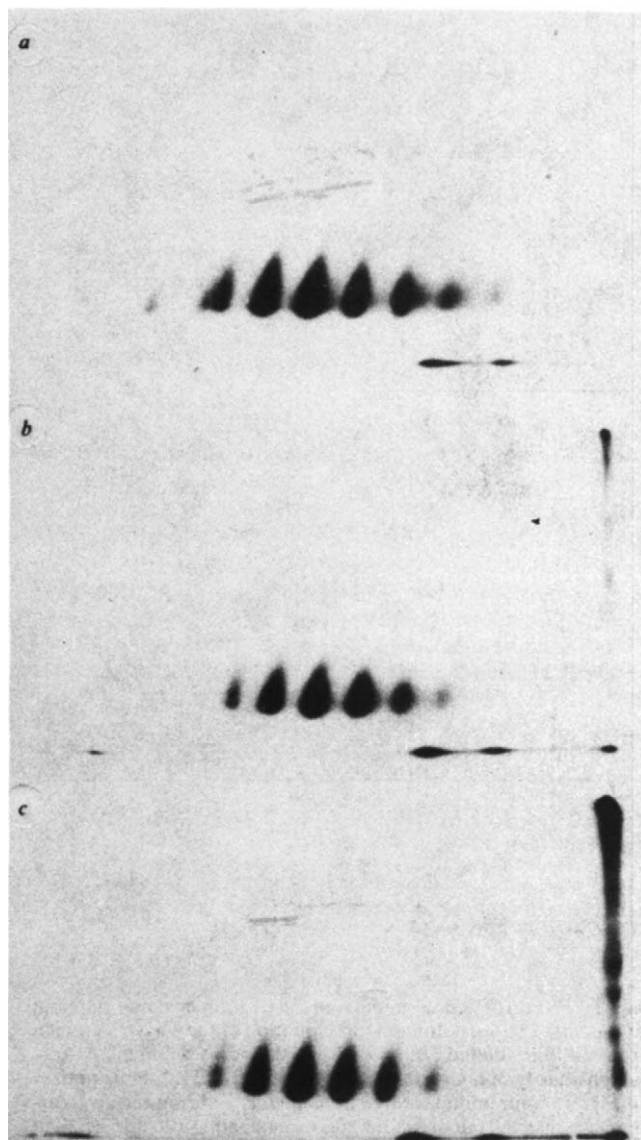


Fig. 2 Two-dimensional mapping of the Thy-1.1 and Thy-1.2 antigens. Thymocytes and mesenteric lymph node lymphocytes of AKR (Thy-1.1) origin were radio-iodinated and lysed as in Fig. 1. Lysates corresponding to 10×10^6 cells were immunoprecipitated⁹ with 5 μ l of anti-MTLA antiserum¹⁰. Immunoprecipitated Thy-1 molecules were mixed with selected isoelectric point and MW markers (m and o) labelled with 35 S-methionine in the isoelectric focusing buffer of O'Farrell and run in NEPHGE⁷ conditions. Second-dimension gels and autoradiography were carried out as in Fig. 1. Only a portion of each autoradiogram is shown, orientated as in Fig. 1. *a*, AKR thymocytes; *b*, BALB/c thymocytes; *c*, AKR mesenteric lymph node lymphocytes; *d*, BALB/c mesenteric lymph node lymphocytes.

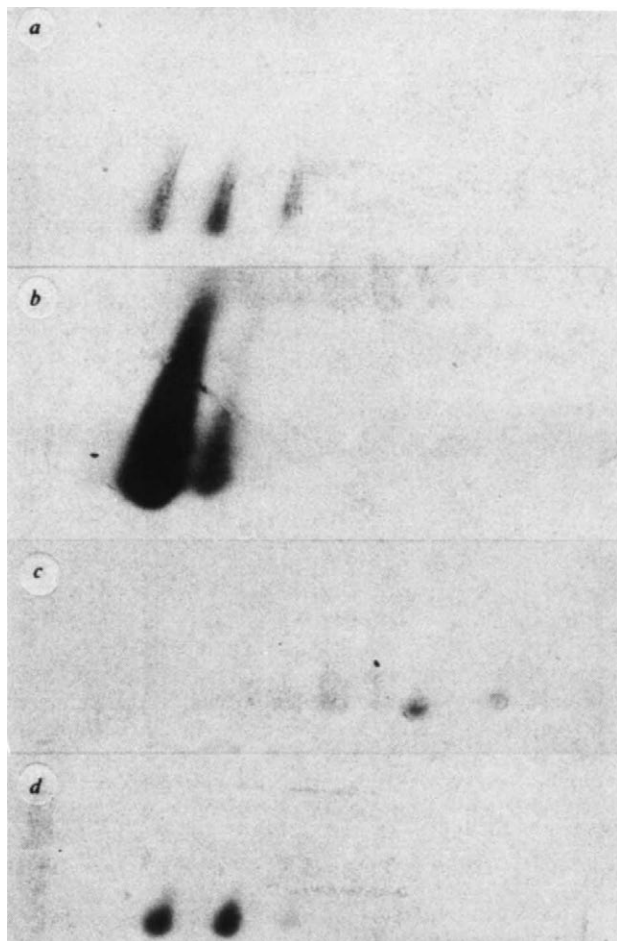


Fig. 3 Effects of neuraminidase on the two-dimensional mapping of the Thy-1 antigen. Immunoprecipitation of the Thy-1.1 antigen from radio-iodinated lysates of AKR thymocytes and mesenteric lymph node lymphocytes was done using anti-MTLA antiserum, as in Fig. 1. Neuraminidase (*Vibrio cholerae*, Behringwerke) treatment was carried out on whole cells by incubating 100×10^6 cells in 2 ml of Tris-HCl 10 mM, pH 6.0, NaCl 0.14 M, CaCl_2 10 mM with 0.1 IU of enzyme per ml for 60 min at 37 °C. Treated cells were washed twice with PBS and radio-iodinated. Neuraminidase treatment in these conditions did not result in a decrease in lymphocyte viability. Second-dimension gels and autoradiography were carried out as in Fig. 1. Portions of autoradiograms shown are aligned on minor neuraminidase-insensitive contaminants in the immunoprecipitate. The basic end of the pH gradient is to the left of each panel. *a*, Thymocytes, untreated; *b*, thymocytes, neuraminidase treated; *c*, mesenteric lymph node lymphocytes, untreated; *d*, mesenteric lymph node lymphocytes, neuraminidase treated.

cules, showing that the charge difference is associated with the *Thy-1* locus. The difference is still observed after neuraminidase treatment, suggesting that there is a charge difference at the amino acid level between AKR and BALB/c Thy-1 molecules. Such an alteration could be responsible for the antigenic difference between the Thy-1.1 (AKR) and the Thy-1.2 (BALB/c) molecules. It has already been suggested that this difference is due to protein rather than carbohydrate polymorphism⁵, but a role for carbohydrate in Thy-1 antigenicity¹⁵ is not excluded.

It is not clear whether changes in the carbohydrate structure of given glycoproteins commonly accompany cellular differentiation. At least one other group of T-cell high MW glycoproteins also undergoes increased sialylation during T-lymphocyte maturation¹⁶, but not every lymphocyte glycopro-

tein is modified when the cell leaves the thymus (D.H., P. Vassalli and J.R.L.P., in preparation). The increased sialylation of the Thy-1 and high MW glycoproteins cannot by itself account for the increased sialic acid content of peripheral T cells, both because peripheral lymphocytes carry less Thy-1 than do thymocytes³, and because the number of Thy-1 and high MW glycoprotein molecules per peripheral cell ($<10^6$)³ cannot completely account for the difference in sialic acid content between the two cell types ($>2 \times 10^7$ molecules per cell)⁶.

The overall increase in sialic acid content of peripheral T cells may be functionally significant. Woodruff and Gesner¹⁷ have shown that neuraminidase-treated peripheral lymphocytes do not home to lymphoid organs when injected intravenously into syngeneic hosts, but are quickly trapped in the liver. Moreover, normal, poorly sialylated thymocytes do not home efficiently to peripheral lymphoid organs¹⁸. Changes in the structure of glycoprotein carbohydrates may be as important for cellular function as the quantitative changes which occur in the amounts of cell-surface glycoproteins during lymphocyte maturation¹⁻⁴.

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Autoreactive natural killer-like cells from agar-cloned murine bone marrow cells

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Natural surveillance against malignant cells has been ascribed to so-called natural killer (NK) cells. These cells attack tumour cells without prior antigen stimulation and may also be cytotoxic to non-malignant cells^{1,2}. NK cells are routinely demonstrated by their cytotoxic activity against various types of tumour cell *in vitro*, but elucidation of the characteristics of the cytotoxic cells has hitherto been hampered by the lack of techniques by which NK cells can be isolated. Here we demonstrate murine NK-like activity from bone marrow cells cloned in semi-solid agar medium for granulocyte-macrophage colony formation. Data obtained indicate that such procedures considerably improve the possibility of obtaining quite pure NK cell populations by which the NK cell phenomenon may be studied in more detail.

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Dose-response data for the cytotoxicity of mass-collected cluster and colony cells from day 7 cultures show (Table 1) that a variety of syngeneic, allogeneic and xenogeneic tumour cells as well as fetal syngeneic fibroblasts are sensitive to cytotoxic attack in a ratio of target:attacker cell as low as 1:0.2 to 1:0.1. The human Burkitt lymphoma cell line (Raji) was resistant to killing, but all other tumour lines examined were sensitive. The most sensitive targets were the syngeneic YAC-1 tumour cell line and the malignant cell line from metastatic foci of the allogeneic Lewis lung tumour model described recently³.

Table 1 Cytotoxicity of mass-collected cluster and colony cells against syngeneic fibroblasts and syngeneic, allogeneic and xenogeneic tumour cell lines

Target cells	Target: attacker cell ratio				
	1:2	1:0.66	1:0.20	1:0.10	1:0.04
Fetal fibroblasts	48%*	39%	38%	20%	NS
YAC-1 (BALB/c)	40%	41%	41%	37%	27%
NS-1 (BALB/c)	27%	20%	21%	26%	NS
BW 5147 (AKR)	16%	19%	18%	NS	NS
Lewis lung (C57B1) metastatic foci	18%	17%	14%	NS	NS
Lewis lung primary tumour	57%	39%	41%	15%	NS
Raji (human) (Burkitt lymphoma)	NS	NS	NS	NS	NS
Molt-3 (human) (T-leukaemia)	21%	21%	24%	NS	NS

The cytotoxic properties of cluster and colony cells, that is, aggregates composed of less or more than 50 cells, from 7-d bone marrow cultures with 2×10^5 cells per ml agar medium⁹. Mice were 2-3 months old BALB/c. Colony-stimulating factor (GM-CSF) was a fivefold concentrated supernatant from concanavalin A stimulated spleen cells¹⁰ used in a concentration of 1.25%. Cluster and colony cells were mass collected by mechanical disruption of the agar in serum-free RPMI-1640 medium. The agar was removed by centrifugation over fetal calf serum (FCS) at 1,000g and the pelleting cells were washed twice and counted for viability before assay. When individual colonies were assayed they were transferred from the agar to 0.4 ml RPMI-1640 medium by a Pasteur pipette and dispersed by pipetting. The cytotoxic properties of the collected cells were tested in a 4-h ⁵¹Cr-release assay¹¹. The malignant cell lines and non-malignant embryonic BALB/c fibroblasts in their second passage were labelled with ⁵¹Cr and used as target cells. Briefly, fivefold replicate cultures of 10^4 target cells in 0.1 ml RPMI-1640 medium labelled with ⁵¹Cr were added to wells of 96-well microplates followed by addition of 0.1 ml attacker cells in appropriate number according to the target:attacker cell ratio. When individual colonies were assayed cultures were run in duplicates and the target cell number was 5,000. The supernatant of each well was collected 4 h later and the ⁵¹Cr content determined. Total release was determined by addition of distilled water to some wells. Spontaneous release was determined by release in wells containing only ⁵¹Cr-labelled target cells. Spontaneous release was 10-15% of total release. s.e. values were about 5-10%. The specific release was calculated as: [(counts in experiment - counts in spontaneous release)/(total release - spontaneous release)] $\times 100$. In fivefold replicate cultures specific release more than 10% was significant at $P < 0.01$ level. In duplicate cultures (assay of single colonies) specific release was considered significant when equal to or more than 20%.

* Specific ⁵¹Cr release, mean of fivefold replicate cultures.

Table 2 shows the kinetics of appearance of cytotoxic activity in relation to duration of culture. The cytotoxic activity was highest on days 6-7 of culture and no autoreactivity was detected on days 4 and 12.

Mass-collected cluster and colony cells obtained on day 7 were separated according to the presence or absence of Fc receptor and the cytotoxicity tested (Table 3). Only the Fc receptor-negative cell population was cytotoxic. Table 3 also shows that mature granulocyte colony cells collected from day 8 cultures were without cytotoxic effect. Similarly, bone marrow cells cultured in liquid medium in the presence of concanavalin A and tested for cytotoxicity after 4-11 d of culture showed no cytotoxic activity. Both mass-collected mouse B-lymphocyte colony cells and human T-lymphocyte colony cells were without cytotoxic activity. Table 3 includes results obtained with normal and activated adherent mouse peritoneal cells. None of these cell categories was cytotoxic. Finally, Table 3 shows that the cytotoxic activity of mass-collected cluster and colony cells from bone marrow cultures was lost by freeze-thawing the attacker cells before assay.

Of 115 single colonies from day 6-7 bone marrow cultures assayed individually (Table 1 legend) for cytotoxicity against both YAC-1 tumour cells and fetal syngeneic fibroblasts, 16 cytotoxic colonies were detected. Of these, 14 were cytotoxic to YAC-1 tumour cells (mean specific ⁵¹Cr release 31%, range 23-40%) and nine of the colonies were cytotoxic to syngeneic fibroblasts (mean specific ⁵¹Cr release 26%, range 20-38%). Seven of the colonies were cytotoxic to both tumour cells and fibroblasts. Four replicate agar cultures containing 2×10^5 bone marrow cells per culture were typed at day 7. Individual colonies were transferred to microscopic slides, fixed in glutaraldehyde (2.5%) and stained with Mayer's haematoxylin followed by Giemsa staining. Of these colonies 66% were of the mononuclear-macrophage type, including the dispersed type of colonies described below, 16% of the colonies were composed mainly of polymorphs and 18% were typical mixed macrophage-granulocyte colonies (Fig. 1). The cultures contained fivefold as many clusters which were not typed.

In separate experiments, morphology and cytotoxicity of individual colonies were studied simultaneously. Half of the colony was fixed and stained as above and the other half was assayed for cytotoxicity. The colony type that most often showed cytotoxic activity appeared in the agar as very dispersed aggregates composed of uniform, small cells (Fig. 1a). The cell number in these colonies at day 7 of culture was 500-1,000. Differential counts of five cytotoxic colonies of the dispersed type revealed that $64 \pm 18\%$ of the colony cells were small, mononuclear cells with a diameter below 10 μ m. The cytoplasm of these cells was pale and contained a few small nonspecific granules but no phagocytosed agar. In contrast, the few mature macrophages ($6 \pm 5\%$) present in these cytotoxic colonies all contained large spheres of phagocytosed agar in their cytoplasm. In all the cytotoxic dispersed colonies a considerable number of polymorphs were present. The dispersed type of cytotoxic colonies constituted 3-4% of all colonies at day 7 of culture. More rarely, cytotoxic cells were present in colonies which appeared in the agar as composed of macrophages only or macrophage-granulocyte mixtures (compare Fig. 1b, c). However, in these colonies the small mononuclear cell type described above was always present in significant numbers.

Table 2 Cytotoxicity of mass-collected cluster and colony cells after different periods of culture against YAC-1 tumour cells and syngeneic embryonic fibroblasts (Fib)

Days of culture	Target: attacker cell ratio									
	1:1		1:0.33		1:0.10		1:0.05		1:0.02	
	YAC-1	Fib	YAC	Fib	YAC	Fib	YAC	Fib	YAC	Fib
4	40%*	NS	20%	NS	NS	NS	NS	NS	NS	NS
5	47%	32%	33%	NS	14%	NS	NS	NS	NS	NS
6	60%	52%	12%	24%	NS	NS	NS	NS	NS	NS
7	58%	33%	35%	34%	26%	NS	NS	NS	NS	NS
9	29%	14%	26%	14%	15%	NS	NS	NS	NS	NS
12	16%	NS	15%	NS	NS	NS	NS	NS	NS	NS

For explanations of technique see Table 1 legend. NS, non-significant ⁵¹Cr release.

* Specific ⁵¹Cr release, mean of quintuplicate cultures.

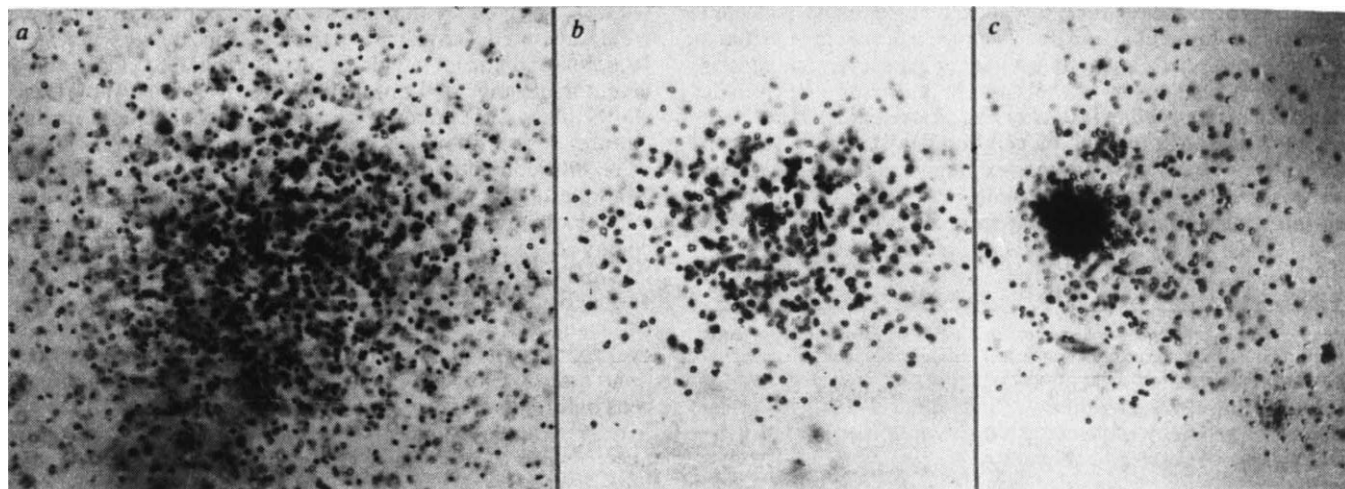


Fig. 1 a, A Colony with cytotoxic activity photographed in the agar medium. Note the uniform size of the very dispersed colony cells. b, A typical macrophage colony. c, A mixed macrophage-granulocyte colony. $\times 15$.

The present results clearly demonstrate that cells with an autocytotoxic effect and with NK-like activity can be grown on a clonal basis from bone marrow cells stimulated by GM-CSF in agar medium. Clones with cytotoxic activity contain in addition to typical polymorphs and macrophages a substantial number of small mononuclear cells. It is known that both macrophages and polymorphs derived from GM colonies possess the Fc receptor⁴,

T lymphocytes⁶, but has been suggested to belong to a pre-T-lymphoid subpopulation¹. The present culture system is presumed only to permit proliferation of non-lymphoid precursor cells, that is, GM-colony forming cells⁷. However, it remains to be proven that non-B, non-T-lymphoid cells cannot proliferate and differentiate to a certain degree (without gaining receptors typical of mature lymphocytes) in the present agar culture system. Therefore, we cannot exclude the possibility that the cells with NK-like activity might be subsets of lymphoid cell precursors.

We have previously shown that activated autoreactive cells possess the Fc receptor³, and recently it was shown that NK cells which had been activated *in vitro* may also be Fc receptor positive⁸. The non-activated cytotoxic cell of the present study was Fc receptor negative. It is therefore possible that NK cells and cytotoxic autoreactive cells not only belong to the same subset of cytotoxic cells but also represent different differential steps eventually within the same clone of cytotoxic cells, as seven out of nine autoreactive colonies detected in the present study also showed cytotoxicity against tumour cells.

In conclusion, it is possible to detect cytotoxic clones by their morphology in agar and it should thus be possible by this technique to obtain highly purified preparations of the cells with both cytotoxic autoreactivity and NK-like activity. This will improve the possibility for a complete phenotypic characterisation of the effector cells and their natural biological function.

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Table 3 Cytotoxicity of different cell populations against YAC-1 tumour cells and syngeneic embryonic fibroblasts (Fib)

Attacker cell	Target: attacker* cell ratio	Target cells	
		YAC-1	Fib
Unseparated GM-cluster and colony cells	1:0.4	41% ⁺	39%
Fc ⁺ fraction†	1:0.4	NS	NS
Fc ⁻ fraction†	1:0.2	25%	22%
Freeze-thawed cluster and colony cells	1:0.4	NS	NS
Mature granulocytic colony cells‡	1:1	NS	NS
Bone marrow cells in liquid culture + Con A, days 4–11 of culture§	1:10	NS	NS
Mouse B-lymphocyte colony cells ¹³	1:1	NS	NS
Human T-lymphocyte colony cells ¹⁴	1:1	NS	NS
Peritoneal adherent cells normal	1:2.5	NS	NS
activated	1:7	NS	NS

* Highest number of attacker cells assayed.

† Prepared according to ref. 12. Separated on Isopaque-Ficoll at 1,700g.

‡ Day 8 GM agar culture. Morphology confirmed microscopically.

§ Bone marrow cells (2×10^6 per ml RPMI-1640 medium + 10% FCS + $5 \mu\text{g}$ concanavalin (Con A)).

|| Mice injected intraperitoneally with thioglycolate 4 d before peritoneal washing and cell collection.

⁺ Specific release, mean of fivefold replicate cultures.

whereas the cytotoxic cell in the present study was Fc receptor negative. Moreover, cytotoxicity was not detected in mature macrophages or polymorphs. Taken together, our results suggest that the cytotoxic cell should be sought among the small mononuclear cells most frequently present in the cytotoxic colonies of the undifferentiated, dispersed type.

Previous studies have shown that mouse NK cells are non-adherent, non-phagocytic cells lacking the Fc receptor for IgG (ref. 5). The NK cell also lacks surface receptors typical for B and

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Direct cloning and extended culture of antigen-specific MHC-restricted, proliferating T lymphocytes

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The development of antigen-specific, T-cell lines has been a subject of intensive investigation by many laboratories interested in the problems of the nature of the T-cell receptor and the mechanism of major histocompatibility complex (MHC) restriction. We describe here a modification of the soft agar cloning technique which has allowed us to rapidly generate and expand proliferating T-lymphocyte clones directly from murine lymph nodes. Using this technique, we demonstrate that such T-cell clones are antigen specific and require I-A compatible antigen-presenting cells for stimulation.

Analysis of the B-cell repertoire of antigen-specific receptors has been greatly facilitated by the availability of monoclonal antibodies from both myeloma tumours¹ and hybridomas². In contrast, little is known about either the constant regions or the antigen-combining properties of the receptors of T lymphocytes. Some progress has been made with the use of anti-idiotypic antibodies as probes for examining individual families of T-cell receptors among whole T-cell populations³ and through direct isolation in small quantities of hapten-binding receptors⁴. Clearly, however, analysis of the T-cell repertoire would be greatly facilitated by the ready availability of large quantities of clonal products. The early observations of Bensasson *et al.*⁵ indicated that specific enrichment of proliferating guinea pig T cells could be achieved using repeated stimulation with antigen-pulsed macrophages. Ruscelli *et al.*⁶ then demonstrated that T lymphocytes from human peripheral blood could be maintained in long-term cultures if the growth medium was supplemented with supernatants from mitogen-stimulated lymphocytes. These studies were extended by Gillis and Smith⁷ who were able to propagate both human and murine cytotoxic T lymphocytes. Recently this approach has been applied to antigen-specific T helper cells to isolate cells specific for keyhole limpet haemocyanin (KLH)⁸ and sheep red blood cells⁹. Although such long-term T-lymphoblast cell lines were enriched for either alloreactive or helper T cells, they did not constitute clones of antigen-specific cells. Sredni *et al.*¹⁰

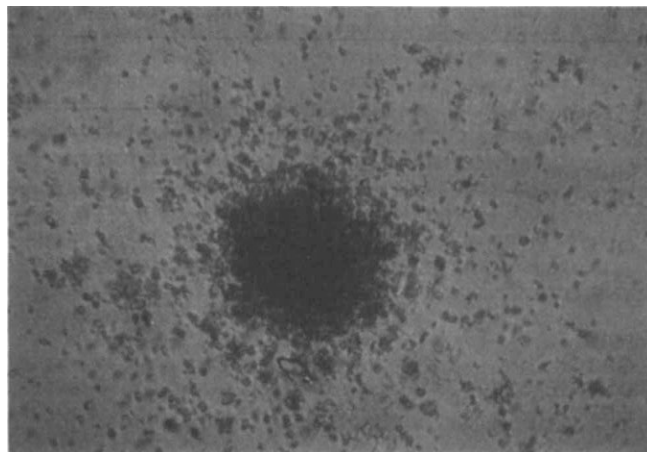


Fig. 1 A colony of proliferating T lymphocytes derived from a single B10.A lymph node cell specific for DNP-OVA. The cells were cloned in soft agar as described in Table 1. After expansion in liquid culture (Table 2) cells from this colony were all shown to be T lymphocytes by indirect immunofluorescent staining on the fluorescence activated cell sorter using a hybridoma anti-Thy.1 reagent* and a fluorescein-labelled rabbit F(ab')₂ anti-mouse immunoglobulin.

developed a technique for cloning mitogen-stimulated T lymphocytes in soft agar and Nabholz *et al.*¹¹ and Fathman and Hengartner¹² used this technique to clone alloreactive murine T cells from long-term T-lymphoblast cell lines; however, the clones often lost the capacity to mediate cytotoxicity. Recently, long-term cytotoxic cell lines have been successfully cloned by limiting dilution techniques¹³. Although these results represent significant advances in the development of monoclonal populations of T lymphocytes, it has not previously been possible to directly isolate from the animal a large number of antigen-specific T-cell clones which could be reliably expanded for analysis of function and specificity.

Primer lymph node cells from C57BL/10 congenic mice bearing different H-2 haplotypes were cloned in soft agar as described in the legend to Table 1. A typical colony is shown in Fig. 1. The number of colonies obtained was similar for all three antigens tested—dinitrophenyl-ovalbumin (DNP-OVA), poly(Glu⁶⁰Ala³⁰Tyr¹⁰) (GAT), and purified protein derivative of tuberculin (PPD). This clonal frequency was approximately 20 to 30 colonies per 2×10^6 cells plated or 1 to 1.5×10^{-5} . However, the colonies elicited by the different antigens were independent of one another as shown in Table 1 by the additive

Table 1 Cloning of proliferating T lymphocytes

Mouse strains	Priming antigen	Antigen <i>in vitro</i>	No. of colonies per 2×10^6 cells seeded*	Clonal frequency
B10	GAT in CFA	GAT	21 ± 3	1.1×10^{-5}
B10	GAT in CFA	PPD	32 ± 3	1.6×10^{-5}
B10	GAT in CFA	GAT+PPD	52 ± 4	2.6×10^{-5}
B10.S	GAT in CFA	GAT	2 ± 0.3	1.0×10^{-6}
B10.S	GAT in CFA	PPD	31 ± 4	1.6×10^{-5}
(B10.S $\times$ B10)F ₁	GAT in DFA	GAT	20 ± 3	1.0×10^{-5}
B10.A	DNP-OVA	DNP-OVA	24 ± 2	1.2×10^{-5}

B10 congenic mice were primed in the hind footpads and at the base of the tail with $50 \mu\text{g ml}^{-1}$ DNP-OVA or GAT in Freund's complete adjuvant (CFA) containing H37Ra¹⁷. Inguinal and popliteal lymph nodes were aseptically removed 7–10 d later. Single cell suspensions were prepared and passed over nylon wool columns^{14,17}. The nonadherent cells (2×10^6) were stimulated in Costar plates (no. 3524) with soluble antigen ($100 \mu\text{g ml}^{-1}$) in the presence of syngeneic irradiated (2,000 R) spleen cells (5×10^5) as a source of presenting cells¹⁶ in a final volume of 1 ml of Click's medium¹⁷ supplemented with 10% heat-inactivated fetal calf serum (FCS). After 3 d of culture the cells were collected, washed and cloned in soft agar in 30-mm Petri dishes¹⁰. The lower agar layer consisted of 2.5 ml of Click's medium supplemented with 20% FCS, 0.5% Noble agar and 20 – $100 \mu\text{g ml}^{-1}$ antigen. The upper layer consisted of 2×10^6 T cells in 0.85 ml of the same medium but containing 0.32% agar. The dishes were incubated at 37°C in a humidified atmosphere with 5% CO_2 for 3–5 d. During this time the majority of seeded cells died while a small number grew out into colonies, each consisting of at least 50 cells. The values presented were represent the arithmetic mean of three separate experiments.

* Mean $\pm$ s.e.m.

Table 2 Antigen specificity of cloned and expanded T cells

Stimulating antigen	Concentration ($\mu\text{g ml}^{-1}$)	Incorporation of ^3H -thymidine (c.p.m.)*		No. of recloned colonies/100 cells seeded*	
		DNP-OVA clone	GAT clone	DNP-OVA clone	GAT clone
Medium	—	29 (± 7)	16 (± 3)	0	0
DNP-OVA	100	7,685 (± 675)	24 (± 4)	74 (± 6)	1 (± 1)
	300	7,714 (± 838)	ND	86 (± 4)	ND
OVA	100	3,774 (± 438)	32 (± 9)	33 (± 3)	0
	300	8,381 (± 929)	ND	82 (± 10)	ND
TNP-OVA	100	3,887 (± 739)	32 (± 9)	37 (± 5)	0
	300	8,765 ($\pm 1,019$)	ND	85 (± 9)	ND
PPD	100	34 (± 7)	37 (± 1)	0	0
GAT	100	39 (± 3)	7,887 (± 930)	0	66 (± 7)
DNP-GL	100	81 (± 45)	260 (± 87)	0	1 (± 1)
DNP-GLA	100	204 (± 106)	30 (± 5)	0	1 (± 1)
TNP-KLH	100	44 (± 8)	33 (± 5)	0	0

Colonies were picked from soft agar using a stereo-microscope. They were placed in flat-bottomed, microtitre wells in Click's medium along with 1×10^4 irradiated syngeneic spleen cells, $100 \mu\text{g ml}^{-1}$ antigen and 20% (v/v) of a supernatant from Con A-stimulated spleen cells prepared as described by Rosenberg *et al.*¹⁸. The clones were expanded in these conditions for 2–3 weeks. Cultures were fed every 3 d by removing 50–70 μl of medium and replacing it with 100 μl of fresh medium containing antigen, irradiated spleen cells and Con A supernatant. After suitable expansion aliquots of the clones were examined for reactivity in a standard 6-d proliferation assay^{14,17} using ^3H -thymidine incorporation (1 to 2×10^3 cells) with supplemental feeding at 3 d or by recloning (200 cells) as described in Table 1 immediately after feeding. The experiments shown were carried out with cells derived from a single clone of B10.A T cells selected for DNP-OVA reactivity or a single clone of B10 T cells selected for GAT reactivity. DNP-poly(Glu⁶⁰Lys⁴⁰)(DNP-GL) and DNP-poly(Glu⁴²Lys²⁸Ala³⁰)(DNP-GLA) were gifts from Dr Charles Janeway, Department of Pathology, Yale University Medical School. The values for thymidine incorporation represent the mean of three separate experiments, the values for recloning the mean of two separate experiments. ND, not determined.

* Means $\pm$ s.e.m.

result obtained when both GAT (21 colonies alone) and PPD (32 colonies alone) were used together (52 colonies). Similar numbers of colonies were obtained from mice bearing different (MHC) haplotypes when antigens to which all strains respond, such as PPD and DNP-OVA, were used. In contrast, strains known to be low responders to particular antigens¹⁴ yielded only a few colonies. For example, C57BL/10 (B10) is a high responder to GAT, whereas B10.S is a low responder. As shown in Table 1, the clonal frequency for GAT of B10 lymph node cells was 1.1×10^{-5} and that for B10.S was only 1×10^{-6} , yet these same two strains gave almost identical clonal frequencies for PPD. The F_1 hybrid between these two strains (B10.S $\times$ B10) behaved like the high responder with a clonal frequency of 1×10^{-5} (Table 1). These results correlate perfectly with the antibody¹⁵ and T lymphocyte proliferative¹⁴ responses to GAT found in these strains and suggest that the MHC-linked immune response (*I*r) genes that control the magnitude of those responses also control the differences in clonal frequency.

Colonies were picked and the cells expanded in liquid culture. Cell growth required antigen, fresh antigen-presenting cells in the form of irradiated spleen cells, and growth factors from 48 h supernatants of concanavalin A (Con A)-stimulated spleen cells (Table 2 legend). More rapid expansion could be achieved if free Con A was also added; however, this was not essential. When the cells had increased in number to 10^5 , individual clones were chosen to study antigen specificity. Specificity was examined by thymidine incorporation and by recloning. As shown in Table 2, a colony originally selected for responsiveness to GAT could only be restimulated by GAT. One thousand cells incorporated enough ^3H -thymidine to give 7,900 c.p.m. whereas the same number of cells stimulated with DNP-OVA or PPD yielded background radioactivity levels. Similarly, only GAT stimulation yielded significant numbers of colonies on recloning, 66 colonies from 100 cells plated for a clonal frequency of 6.6×10^{-1} . On the other hand a colony originally selected for responsiveness to DNP-OVA could not be stimulated by GAT or

Table 3 Control of antigen presentation by genes in the I-A subregion of the MHC

Spleen cells		T-lymphocyte clones (source and specificity)		
Strain	MHC*	Presence of antigen	B10.A: specific for DNP-OVA	B10: specific for GAT
^3H -thymidine incorporation (c.p.m.)				
B10.A	kkkkkddd	—	34 (± 1)	19 (± 5)
		+	16,475 ($\pm 1,379$)	19 (± 4)
B10	bbbbbbbbb	—	32 (± 5)	27 (± 5)
		+	30 (± 9)	21,797 ($\pm 1,897$)
B10.A(5R)	bbbkddddd	—	32 (± 2)	25 (± 1)
		+	36 (± 6)	17,953 ($\pm 1,661$)
B10.A(4R)	kkbbbbbbb	—	35 (± 3)	25 (± 3)
		+	13,539 (± 935)	27 (± 2)
A.TL	skkkkkkkd	—	31 (± 5)	15 (± 4)
		+	13,943 (± 403)	20 (± 4)
None		—	31 (± 3)	18 (± 5)
		+	46 (± 6)	26 (± 7)

Irradiated nonimmune spleen cells from various H-2 congenic mouse strains were tested in a 6-d proliferation assay for their ability to present antigen to 2×10^3 DNP-OVA-specific, cloned B10.A T cells or 2×10^3 GAT-specific, cloned B10 T cells. $100 \mu\text{g ml}^{-1}$ DNP-OVA was used to stimulate the B10.A clone and $100 \mu\text{g ml}^{-1}$ GAT was used to stimulate the B10 clone. The conditions were the same as those described in Table 2. The values shown are from one of four such experiments. The standard errors of the mean were calculated from triplicate determinations for each value.

*The letters refer to the haplotype source of origin of the K, I-A, I-B, I-J, I-E, I-C, S, G and D regions of the murine major histocompatibility complex (MHC).

PPD. It could be stimulated by DNP-OVA, TNP-OVA and OVA although not by the hapten on different carriers DNP-poly(Glu⁶⁰Lys⁴⁰) and DNP-poly(Glu⁴²Lys²⁸Ala³⁰), nor trinitrophenyl-keyhole limpet haemocyanin (TNP-KLH). The original carrier alone, OVA, was not as potent a stimulator as DNP-OVA although at high concentrations (300 µg ml⁻¹) it could stimulate as many colonies as the immunogen. This difference is reflected in the complete dose-response curve (data not shown) suggesting that the T-cell receptor has a slightly higher affinity for DNP-OVA than for OVA. These results indicate that the receptor is not uniquely specific for a new antigenic determinant created by the hapten-carrier conjugation but rather that the receptor recognises predominantly a carrier determinant which has been modified by the hapten.

The recloning frequency was also dependent on the concentration of OVA used. At 100 µg ml⁻¹, the clonal frequency was 35 per 100 cells plated (3.5×10^{-1}) whereas at 300 µg ml⁻¹ it reached 80–85 per 100 (8.5×10^{-1}), a level comparable to that achieved with DNP-OVA. These high recloning frequencies represent an enrichment in specific cells of 70,000-fold relative to the clonal frequency obtained with the cells freshly removed from the animal (compare Tables 1 and 2). This result strongly suggests that our single step technique has produced true T-cell clones, and that the recloning frequencies actually represent the highest attainable cloning efficiencies of the method. To prove that the colonies were really derived from a single cell, 14 of the recloned DNP-OVA colonies were expanded and tested for antigen specificity. All of them showed a pattern of responsiveness identical to that shown in Table 2 for the original DNP-OVA colony. As all the progeny are identical it is reasonable to assume that they have been derived from a single homogeneous population, that is, that the original colony was a true clone.

The requirement for fresh irradiated spleen cells to expand the clones in liquid culture suggested antigen-presenting cells were necessary to effectively stimulate the T lymphocytes. Previous studies¹⁶ from this laboratory have demonstrated that the genes encoded in the I-A subregion of the MHC control such T lymphocyte antigen-presenting cell interactions. Table 3 shows that the same restrictions apply to the clones. A GAT-specific clone isolated from a B10 mouse and a DNP-OVA-specific clone isolated from a B10.A mouse were stimulated with their respective antigens in the presence of irradiated spleen cells from either B10 or B10.A mice or mice bearing recombinant chromosomes in the MHC. In the absence of antigen no stimulation was observed (Table 3), even if syngeneic and allogeneic spleen cells were added together or F₁ spleen cells were used (data not shown). This absence of a mixed lymphocyte reaction indicates that these two particular clones do not possess allo-receptors for B10 or B10.A MHC gene products. In the presence of antigen only spleen cells histocompatible at the I-A subregion supported a proliferative response. Thus, the DNP-OVA-specific B10.A clone was stimulated by antigen in the presence of B10.A, B10.A(4R) and A.TL spleen cells but not B10 or B10.A(5R) spleen cells. For the GAT-specific B10 clone the results were just the opposite (Table 3). These genetic results are identical to what we have previously reported for whole T-cell populations¹⁶. They demonstrate that the phenomenon of MHC restriction is not the result of suppression by cells other than the responding cells, but rather a failure of individual clones to respond to the antigen unless it is presented in association with the appropriate MHC products.

The method we have described provides a rapid and direct technique for the generation and expansion of antigen-specific T-lymphocyte clones. Several of these clones have been maintained in culture for 4 months without loss of specificity. The power of this method is the ability to obtain directly from the animal pure populations of cells which are present in relatively low frequency (for example, for GAT the precursor frequency = clonal frequency ÷ plating efficiency = 3 per 10⁵, assuming that the recloning frequency represents the plating efficiency). Thus, any problems of *in vitro* selection inherent in the techniques of

cloning long-term lymphoblast cell lines^{11–13} are avoided. These clones should prove useful for the biochemical analysis of T-lymphocyte function, for the purification and characterisation of T-lymphocyte receptors and for the study of the mechanism of MHC restriction and the action of *Ir* genes.

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A monoclonal antibody to human acute lymphoblastic leukaemia antigen

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Previous studies by Greaves¹ and others^{2–6} have demonstrated the existence of an antigen associated with cells from many patients with acute lymphoblastic leukaemia (ALL) and some patients with chronic myelocytic leukaemia (CML) in blast crisis. Antisera to this common ALL antigen (CALLA) have been produced in rabbits and require extensive absorption which limits both the titre and quantity of antisera that can be generated and may result in variable specificity in different laboratories. The method for generation of specific antibody by somatic cell hybridisation introduced by Kohler and Milstein⁷ has been successfully used to produce monoclonal antibodies against various normal human cell-surface proteins, including β_2 microglobulin⁸, histocompatibility antigens⁹, thymocyte and peripheral T-cell antigens^{10–12} and Ia-like antigens¹³. The present report describes the generation and characterisation of a monoclonal antibody specific for a common ALL antigen (CALLA) previously identified by conventional heteroantisera.

Our cell fusion procedure was derived from the method developed by Kohler and Milstein¹⁴ with several modifications described by Kennett *et al.*¹⁵. The MOPC-21 variant, P3/NS1/1-Ag4-1, was used as the parent myeloma cell line. Spleen cells were obtained from two BALB/c mice which had each been immunised by intraperitoneal and subcutaneous injections of 2×10^7 ALL cells emulsified in complete Freund's adjuvant. One and five weeks later, an additional 1×10^7 leukaemic cells were injected intravenously. Four days after the last injection, 2.2×10^8 spleen cells were collected and fused with 6×10^7 myeloma cells in the presence of 30% polyethylene glycol and aliquoted into seven microtitre plates (Falcon). Aminopterin was added 24 h after fusion and macroscopic hybridoma colonies became evident between 10 and 28 d in culture.

Rabbit antiserum to CALLA was generated in our laboratory¹⁶ by immunisation with either ALL cells or the Null-ALL cell line Laz 221 (ref. 17), followed by absorption with cells from an autologous B-lymphoblastoid cell line and the leukaemic T-cell line, CEM¹⁸. The initial screening of hybridoma supernatants also used these cell lines, which provided large homogeneous populations of cells that had been extensively characterised with conventional heteroantisera. All cell surface characterisation was done by standard indirect immunofluorescence assay with subsequent analysis of each population by the fluorescence activated cell sorter (FACS-I) (Becton, Dickinson).

Although mice were immunised with cryopreserved ALL cells from a single patient known to be CALLA positive, hybridoma supernatants were initially screened for reactivity to the Null-ALL cell line Laz 221 to select against antibodies to clonotypic antigens. Only supernatants reactive with Laz 221 underwent further analysis with Laz 388 (a B-lymphoblastoid cell line from the same individual as Laz 221) and CEM, thereby approximating the absorptive method for production of rabbit anti-CALLA. Hybridomas reactive with Laz 221 but unreactive with Laz 388 and CEM demonstrated 'CALLA-like' specificity and were then subcultured and injected into pristane-primed BALB/c mice to produce immune ascites¹⁵. These ascites were then screened for reactivity against a large panel of cryopreserved leukaemic cells which had been previously phenotyped with rabbit anti-CALLA. Twenty-two different hybridoma supernatants demonstrated CALLA-like reactivity with

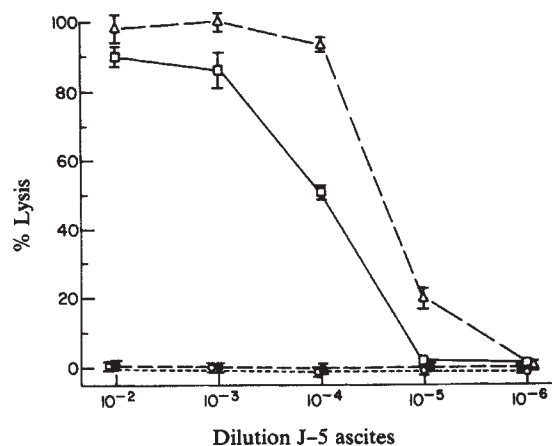


Fig. 2 Specificity of monoclonal J-5 antibody was tested using complement-mediated lysis of ⁵¹Cr-labelled target cells. 4×10^3 radiolabelled target cells (20 μ l) were incubated with various dilutions of J-5 ascites (20 μ l) at 20 °C for 60 min followed by addition of rabbit complement (20 μ l, 1:5 dilution) and further incubation at 37 °C for 60 min. Cell lysis was detected by release of ⁵¹Cr into cell supernatants. All assays were done in triplicate and per cent lysis was calculated as follows: % lysis = [(experimental - spontaneous release)/(freeze-thaw - spontaneous release)] $\times$ 100. $\square$, Laz 221; $\triangle$, NALM-1; $\bullet$, Laz 388; $\circ$, CEM.

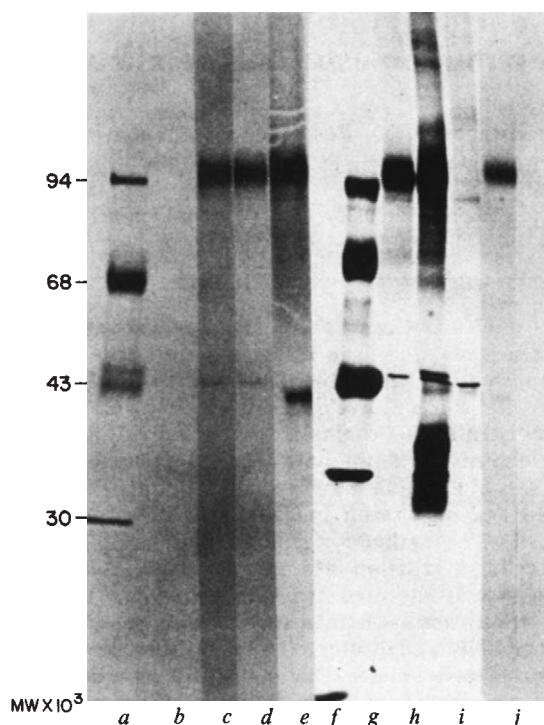


Fig. 1 Immune precipitation experiments with J-5 and rabbit anti-CALLA. *a*, ¹⁴C-labelled MW standards (NEN); *b*, control monoclonal ascites with Laz 221; *c*, J-5 ascites with Laz 221; *d*, J-5 with cells from a CALLA-positive patient; *e*, J-5 with NALM-1; *f*, ¹⁴C-labelled MW standards; *g*, rabbit anti-CALLA with Laz 221; *h*, rabbit anti-ALL serum absorbed only with CEM cells; *i*, normal rabbit serum with Laz 221; *j*, rabbit anti-CALLA with cells from a CALLA-positive patient. Cells were labelled with ³⁵S-methionine (NEN) for 16 h at 37 °C. Nonidet-P40-solubilised membrane extracts were applied to a lentil lectin column and bound glycoproteins eluted with 3% α -methyl-D-mannoside. Peak tubes were pooled and divided equally among samples containing antibody bound to Protein A-Sepharose (Pharmacia). After overnight incubations at 4 °C, samples were washed, heated for 5 min at 110 °C and applied to a large 7–20% gradient polyacrylamide slab gel for electrophoresis at 150 V for 18 h. Autoradiographs of dried gels were obtained by exposure for 7 d at –70 °C.

Laz 221, Laz 388 and CEM, but only one clone had specificity for CALLA when tested with this panel of leukaemic cells. All other clones were reactive with cells from some patients with AML-, CLL-, or CALLA-negative ALL. Table 1 summarises the pattern of reactivity observed with a single hybridoma, J-5, compared with rabbit anti-CALLA. In all 70 populations, reactivity was similar to that of rabbit anti-CALLA demonstrating specificity for leukaemic cells from most patients with non-T-cell ALL and some patients with CML in blast crisis. In addition, no J-5 reactivity was detected with cells from five T-ALL cell lines (CEM, HSB-2, Laz 191, HJD-1 and HHM-1).

Previous studies in several laboratories have demonstrated that heteroantisera with CALLA specificity identify a single glycoprotein with a molecular weight (MW) of 95–100,000 present on some ALL cells and the cell line NALM-1 derived from a patient with CML in blast crisis^{4,19}. Figure 1 demonstrates that immune precipitates with both clone J-5 and rabbit anti-CALLA identify a single 95,000-MW glycoprotein band on cell membranes from Laz 221, NALM-1 and a CALLA-positive patient with ALL, confirming that the two antisera have similar specificity.

Specific reactivity of clone J-5 ascites was also tested by microcytotoxicity assay measuring release of ⁵¹Cr from labelled target cells (Fig. 2). Greater than 50% lysis was observed with ascites dilutions of 10^{-4} on the positive cell lines Laz 221 and NALM-1, but no lysis was noted at any dilution on the negative cell lines Laz 388 and CEM. In experiments designed to test the limits of specificity and effective cytotoxicity of J-5, constant numbers of ⁵¹Cr-labelled Laz 221 cells were mixed with unlabelled autologous cells (Laz 388) before addition of immune ascites (Fig. 3). Significant cytotoxicity was demonstrated after a single treatment with J-5 ascites (1:1,000 dilution), when positive cells represented only 0.1% of the population mixture.

Various haematopoietic cells have also been screened for reactivity with J-5 ascites to determine whether this antibody might be identifying an antigen present on normal cells. No J-5 reactivity was demonstrated with normal peripheral blood mononuclear cells, purified T or B cells, concanavalin A-activated T cells, monocytes or spleen lymphocytes. Whereas Greaves²⁰ and Janossy *et al.*²¹ have reported that CALLA-positive cells are present in fetal and regenerating bone marrow, we have been unable to demonstrate similar reactivity with

Table 1 Reactivity of hybridoma J-5 with leukaemic cells compared to rabbit anti-CALLA

Patients	Rabbit anti-CALLA	Hybridoma J-5
Non-T-cell ALL	21/34*	21/34
T-cell ALL	0/8	0/8
CLL	2/10†	0/10
AML	0/10	0/10
CML-blast crisis	3/5	3/5
CML-stable phase	0/3	0/3

All analyses were carried out on cryopreserved leukaemic cells obtained before the start of chemotherapy, using a standard indirect immunofluorescence assay. 10^6 cells were incubated for 30 min at 4°C with either 0.1 ml J-5 ascites or negative control ascites (1:100 dilution to ensure antibody excess), washed twice, incubated for an additional 30 min at 4°C with 0.1 ml fluorescein-conjugated goat anti-mouse IgG (Melyo, 1:40 dilution), and washed three times before analysis. Intensity of fluorescence with J-5 was determined for 40,000 cells in each population by the FACS-I and compared with fluorescence with negative control ascites. Any reactivity with monoclonal antibody above background was considered positive. Analysis with rabbit anti-CALLA was similar except that antisera were used at a 1:20 dilution and F(ab')₂ fluoresceinated goat anti-rabbit F(ab')₂ was used as a developing reagent.

* Each fraction represents the number of positive populations over the total number tested. Except as described below (†), leukaemic cells were either positive or negative with both rabbit anti-CALLA and hybridoma J-5. Intensity of fluorescence of positive populations, however, was not always identical and cells from three patients exhibited strong fluorescence with rabbit anti-CALLA but only weak reactivity with monoclonal J-5.

† Cells from two CLL patients were initially weakly positive with rabbit anti-CALLA but subsequent cross-absorption experiments¹⁶ demonstrated that this reactivity was not specific for CALLA. Monoclonal J-5 antibody confirms this finding.

monoclonal J-5 antibody using both the FACS-1 and fluorescence microscopy. Eight bone marrow samples have been analysed including normal adult marrow (two volunteers) and regenerating marrow after either chemotherapy (four patients) or bone marrow transplantation (two patients). In addition, there was no detectable reactivity with J-5 ascites and 18-week fetal liver, bone marrow or thymus. This lack of reactivity with regenerating bone marrow and fetal haematopoietic tissues suggests that J-5 antibody is detecting a unique antigen which may be leukaemia specific.

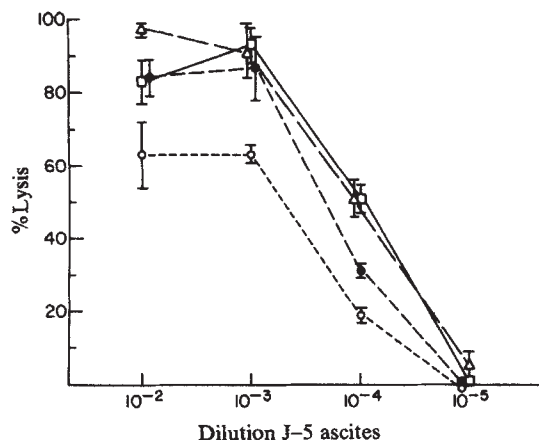


Fig. 3 Effective cytotoxicity of monoclonal J-5 was tested by mixing 10^3 ⁵¹Cr-labelled Laz 221 cells with 10^4 , 10^5 or 10^6 unlabelled Laz 388 cells before addition of ascites at various dilutions. Cells (100 µl) were incubated with ascites (100 µl) at 20°C for 60 min followed by addition of rabbit complement (100 µl, 1:5 dilution) and additional incubation at 37°C for 60 min. All assays were carried out in triplicate and per cent lysis calculated as described in Fig. 2 legend. □, 100% Laz 221; △, 10% Laz 221; ●, 1% Laz 221; ○, 0.1% Laz 221.

These studies demonstrate that J-5 monoclonal antibody will be a valuable diagnostic and therapeutic reagent. The extensive absorption required to make conventional heteroantisera specific for ALL cells and the relatively weak lytic capacity of these reagents have limited their clinical application. Development of a monoclonal antibody with CALLA specificity overcomes these obstacles and will allow clinical trials of *in vitro* and *in vivo* specific immunotherapy.

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The appearance of single-ion channels in unmodified lipid bilayer membranes at the phase transition temperature

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Ion-specific channels in artificial membranes have been formed by the addition of gramicidin A¹, alamethicin², polyene antibiotics³ and some proteins⁴ to the solution surrounding the bilayer lipid membrane. Until now there have been no reports of single-ion channels in unmodified lipid membranes. We have now studied the electrical conductance of planar lipid bilayers membranes made of synthetic distearoylphosphorylcholine (DSPC). Current fluctuations of amplitude ~1 pA and duration ~1 s have been discovered at phase transition temperature, which shows that the appearance of ionic channels may be the result of lipid domain interactions. This would explain the dramatic increase in ion permeability observed in liposomes during phase transition. We suggest that these channels could conduct the transmembrane ionic current in biological membranes without the involvement of peptides and proteins.

The ionic permeability is known to change in the black lipid membrane formed from DSPC at the phase transition⁵, since there is a jump in membrane conductance at 59°C, the phase transition temperature of DSPC. As the change of the integral conductance at this temperature was sharp and reversible, it was suggested that there might be a single-ion channel in the bilayer lipid membrane at this temperature.

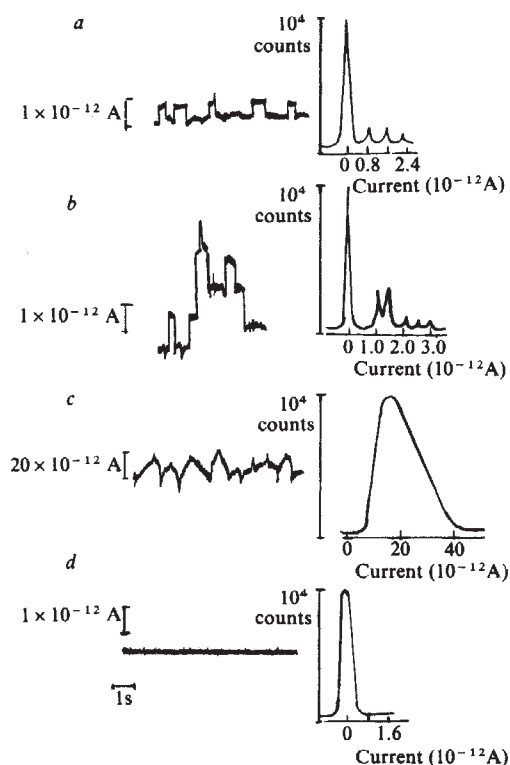


Fig. 1 Left, groups of fluctuations in the current through a lipid bilayer membrane. Right, occurrence of corresponding frequencies of the open channels, recorded by NTA-1024 multichannel analyser. The frequency was obtained by sampling the current at 200- μ s intervals. [Applied potential, 100 mV.] *a*, Bilayer lipid membranes were formed from 0.2% DSPC at $59 \pm 0.2^\circ\text{C}$. The bathing medium contained 1M KCl, 0.003M Tris-HCl pH 7.4. *b*, Bilayer membranes formed from egg lecithin in *n*-decane at 22°C . Gramicidin A 10^{-9} M was added. Egg lecithin was obtained by the method of Folch *et al.*¹². *c*, Bilayer membranes formed from DSPC in *n*-decane at a temperature below 59°C . *d*, Bilayer membranes formed from DSPC in *n*-decane at a temperature above 59°C .

In an attempt to demonstrate this single-ion channel, transmembrane currents were measured in voltage-clamp conditions. The amplitude analysis of the current pulses was produced by multichannel analyser NTA-1024.

The lipid bilayer membranes were formed at holes in a Teflon 'wall' according to Mueller *et al.*⁶ The temperature in the cell was kept constant by a thermostat, and the temperature around the membrane was measured by thermocouple. The DSPC used in this study was synthesised in our laboratory and contained no impurities that could be detected by thin-layer chromatography and other methods⁷. Gramicidin A (Calbiochem) was used without additional purification.

The experiments demonstrated transmembrane current fluctuation when the temperature in the cuvette was near the phase transition temperature of DSPC (Fig. 1*a*, left). The histogram reveals the discrete character of the current impulses distribution (Fig. 1*a*, right). The current fluctuation amplitude ranges discretely from 0.8 to 2.4 pA. Note that if the temperature rises above 59°C , there are no transmembrane current fluctuations and, simultaneously, the integral membrane conductivity falls to 10^{-8} S cm^{-2} . If a noise with amplitude much higher than that of a separate current fluctuation appears, the temperature is well below the phase transition temperature (Fig. 1*c*). The integral conductance of the membrane increases sharply at this temperature. Note that these current changes are completely reversible and may repeat themselves in successive cycles of high and low temperature.

It should also be noted that the appearance of single current fluctuations (Fig. 1*a*, left) was observed in every experiment, but because of technical difficulties, it was not possible in every experiment to record sufficient fluctuations for statistical treatment (Fig. 1*a*, right) (for example, 100 single fluctuations were observed only in 10% of the experiments).

Compared to published data, it is evident that the characteristics of the current fluctuations at the phase transition are very different from those which are obtained by alamethicin² and EIM⁴ on lipid membranes; but life times and amplitudes of the channels formed by gramicidin A in the black lipid membrane are similar (compare Fig. 1*a* and *b*).

Interestingly, the distribution of current fluctuations at the transition temperature of DSPC depends strongly on the length of time that membranes are at the fixed temperature.

The transmembrane current fluctuations reported here are evidence of the appearance of ion channels in non-modified bilayer membranes and prove that the observed ionic channels at the transition temperatures of DSPC are connected with phase transition. We assume that the ion channels appear when lipid domains interact in the membranes.

One possibility that must be considered is the involvement of impurities¹⁰ in channel formation. This seems unlikely for the following reasons. If the channel appearance were due to impurities, we would expect a decrease in integral conductance, infrequent current fluctuation, and displacement of histogram peaks to low-amplitude values, because after repeated temperature transitions the accumulation of impurities at the phase transition should be associated with a release of some impurities into the water solvent. Therefore the content of impurities would be considerably decreased in the lipid bilayer after several repetitions of the cycle. This suggestion is based on the well known effect of zonal rectification of metals from impurities. Published data supporting this approach prove that the constant of distribution of impurities between gel and liquid crystalline phases is rather low (0.029 for dipalmitoyllecithin⁸). However, experimental data showed that neither current fluctuations nor the histogram were changed by repeated phase transitions. The integral conductance scatter is not more than 30%.

During studies of the ionic selectivity of channels we synthesised a positively charged analogue of DSPC containing a P-CH₃ group instead of P-OH group⁹. We suppose that channel walls are formed from polar groups of phospholipids. Neutral DSPC forms channels which show no ionic selectivity, whereas a positively charged analogue induces anion selectivity. This result supports the conclusion that the main phospholipids, and not impurities, are responsible for the ionic channel formation, because the synthesis schemes for the two phospholipids are the same, so the two lipid bilayers should contain identical impurities.

In bilayer membranes formed from a mixture of DSPC with 1 mol % of other synthetic lipids or egg lecithin, at the transition temperature only the DSPC-channels were recorded (data not shown). Thus lipids such as egg lecithin which constitute an impurity do not influence the formation of ionic channels at the phase transition temperature of DSPC. The suggestion impurities do not influence the formation of ionic channels is further supported by data obtained by several authors^{10,11} in studies of ionic selectivity of synthetic phospholipid liposomes at the phase transition.

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Light- and GTP-regulated interaction of GTPase and other proteins with bovine photoreceptor membranes

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Light absorption by photoreceptor rod outer segments (ROS) leads not only to spectral and structural changes in the rhodopsin molecule¹ but also to the activation of several enzymatic activities²⁻⁶ including GTPase⁷⁻¹⁰. The mechanism of this effect is not known; however, in all cases where the action spectrum of light-induced enzyme activation has been measured (see, for example, refs 4, 8), it matched the absorption spectrum of rhodopsin. This suggests that bleaching of rhodopsin is the primary step in enzyme activation, and that some light-induced changes in the molecular interaction of the enzymes with rhodopsin, the major intrinsic ROS membrane protein, should be involved. I have indeed found that light induces profound changes in the interaction of rhodopsin kinase¹¹, GTPase (reported here), and other proteins with the photoreceptor membrane. These changes are reversible in the dark, are strongly influenced by GTP, and are thought to be involved in the regulation of enzyme activity by light. The light-induced binding of the GTPase and its subsequent elution with GTP was used to purify this enzyme.

Bovine ROSs were purified from freshly dissected retinas by flotation and subsequent centrifugation on stepwise sucrose density gradients in 70 mM phosphate buffer¹¹. Electron microscopy¹² revealed large fragments of ROS, 2–20 μ m long, surrounded by a plasma membrane which looked continuous. There was no detectable contamination by other cell fragments. The ratio A_{280}/A_{500} of the ROS was 2.0–2.4, indicating good purity¹³.

Gel electrophoresis of such purified ROSs (Fig. 1a, b) shows that rhodopsin is the major protein but that a number of additional polypeptides is also present, in agreement with published data¹⁴. Most of these additional polypeptides are soluble in the dark in hypotonic buffer (Fig. 1d); little protein besides rhodopsin remains with the washed membranes (Fig. 1c). The major four polypeptides present in dark extracts (doublet bands at molecular weights (MWs) 37,000 and 35,000 and at about 95,000) are extractable at low ionic strength but are barely extractable at moderate ionic strength (70 mM phosphate buffer¹¹, 100 mM Tris buffer, or 120 mM KCl Ringer's); this suggests that they are peripherally bound membrane proteins. Most of the other polypeptides can be extracted at moderate ionic strength, provided the ROS plasma membrane is disrupted, and may therefore be cytoplasmic soluble proteins or only very weakly bound membrane proteins¹¹.

Illumination of the ROS suspension before centrifugation causes certain polypeptides to disappear almost completely from the soluble supernatant (Fig. 1e; MWs 35,000, 37,000, 48,000 and 68,000, and a peptide of MW about 6,000 discernible in the front band near the bottom of the gels). These polypeptides become membrane-bound upon illumination and sediment with the bleached membranes. They are found in the membranous pellet (not shown in Fig. 1) and can be eluted from the pellet by subsequent treatments which is demonstrated, for example, in Fig. 1o, p for the polypeptides of MWs 37,000, 35,000 and 6,000. The light-induced binding to the bleached membranes is slowly reversed in the dark: If ROS suspensions are bleached but then further incubated in the dark for 70 min at 20 °C, then most of the initially bound polypeptides are found again in the soluble supernatant after centrifugation (Fig. 1g).

This light-induced and reversible binding has been observed previously¹¹ for the two polypeptides (MWs 68,000 and 48,000) which are soluble at moderate ionic strength, and the polypeptide of MW 68,000 has been identified as rhodopsin kinase. To demonstrate that the three additional polypeptides (MWs 37,000, 35,000 and 6,000) undergo light-induced changes in their interaction with the membrane, it is necessary to subject the membrane to conditions of low ionic strength. Then, light-dark differences in the binding affinity of these peripheral membrane proteins to the membrane become obvious. The binding is too weak in the dark to withstand the low ionic strength treatment; in light, however, the binding becomes much stronger, such that it resists the low ionic strength treatment for a limited time after bleaching.

Table 1 GTPase activities of the preparations shown in Fig. 1

Gel (Fig. 1)	Preparation	GTPase activity*
a	Purified ROS before extraction, 2.4 μ g rhodopsin	6.0
b	As a, but 12 μ g rhodopsin	28.8
c	Extensively washed ROS membranes, 12 μ g rhodopsin	0.36
d	Dark extract, obtained from unbleached ROS (corresponding to 35 μ g rhodopsin) in 5 mM Tris	41.4
e	Light extract from ROS (35 μ g) bleached for 3 min, centrifuged immediately after bleaching	0.9
f	Extract from bleached ROS, centrifuged after a 2-min dark incubation period at 20 °C following bleaching	1.7
g	Extract from bleached ROS, centrifuged after a 70-min dark incubation period following bleaching	27.1
d'	Dark extract d assayed without addition of washed ROS	0.43
	<i>Effect of GTP on the light-induced binding</i>	
h	Dark extract obtained from unbleached ROS in the presence of 1 mM GTP	—
i	Light extract from ROS bleached in presence of GTP and centrifuged immediately after bleaching	—
k	Extract from ROS bleached in absence of GTP and then dark-incubated for 2 min after adding GTP	—
l	Extract from ROS bleached in absence of GTP and then dark-incubated for 70 min after adding GTP	—
	<i>Purification of the GTPase via light-induced binding</i>	
m	Extract obtained in a second wash of a bleached ROS pellet with 5 mM Tris	1.2
n	Further extraction of the bleached pellet by prolonged incubation with 100 mM Tris at 20 °C	0.22
o	Purified GTPase (2.8 μ g protein) obtained by extracting the thoroughly washed bleached pellet with 40 μ M GTP	38.0
p	Purified GTPase as o, but 13.8 μ g protein	—

The preparations and amounts of protein used for the GTPase assays were the same as for the correspondingly lettered gels in Fig. 1. For details of the extraction procedure in darkness and light see legend to Fig. 1. The GTPase activity values given are averages of four determinations with s.d. < 5%. Assays were performed according to the method of Neufeld and Levy²⁰ with some modifications^{8,15}. Incubation was for 2–8 min at 30 °C in continuous light. The assay mixture contained 2 μ M [γ -³²P]GTP (except sample o, 3 μ M ³²P-labelled GTP) of specific activity 2,200–3,000 c.p.m. pmol⁻¹; 20 mM Tris-HCl (pH 7.4); 5 mM MgCl₂; 1 mM dithiothreitol; and the appropriate amounts of ROS and/or extracts, in a final volume of 200 μ l. To each assay of extracts (d–g, and m–o), 4.7 μ g rhodopsin in the form of washed, GTPase-depleted ROS membranes was added for activation of the extracted GTPase. When GTPase-containing extracts were assayed in the absence of rhodopsin, they showed no GTPase activity (sample d').

* GTPase activity is expressed as pmol P_i liberated per min at 30 °C per amount of protein seen on the gels of Fig. 1.

One of the most striking features of this light-dependent protein binding is that GTP (10^{-5} – 10^{-3} M) inhibits, or reverses, the light-induced binding of the polypeptides MWs 37,000, 35,000 and 6,000 if it is present during bleaching (Fig. 1i) or added shortly after bleaching (Fig. 1k, l). In the dark, GTP has little influence on the extractability in hypotonic buffer (Fig. 1h). This solubilising effect is relatively specific for GTP—other nucleotides had only a partial effect (ATP, guanylyl imidodiphosphate) or no effect (3',5'-cyclic GMP, 5'-GMP, and others)¹⁵. GTP also affects the solubility of the polypeptide of MW 48,000, but in an opposite way: The light-induced binding of this polypeptide, which is normally reversible in the dark (Fig. 1g), becomes irreversible if GTP is present (Fig. 1l).

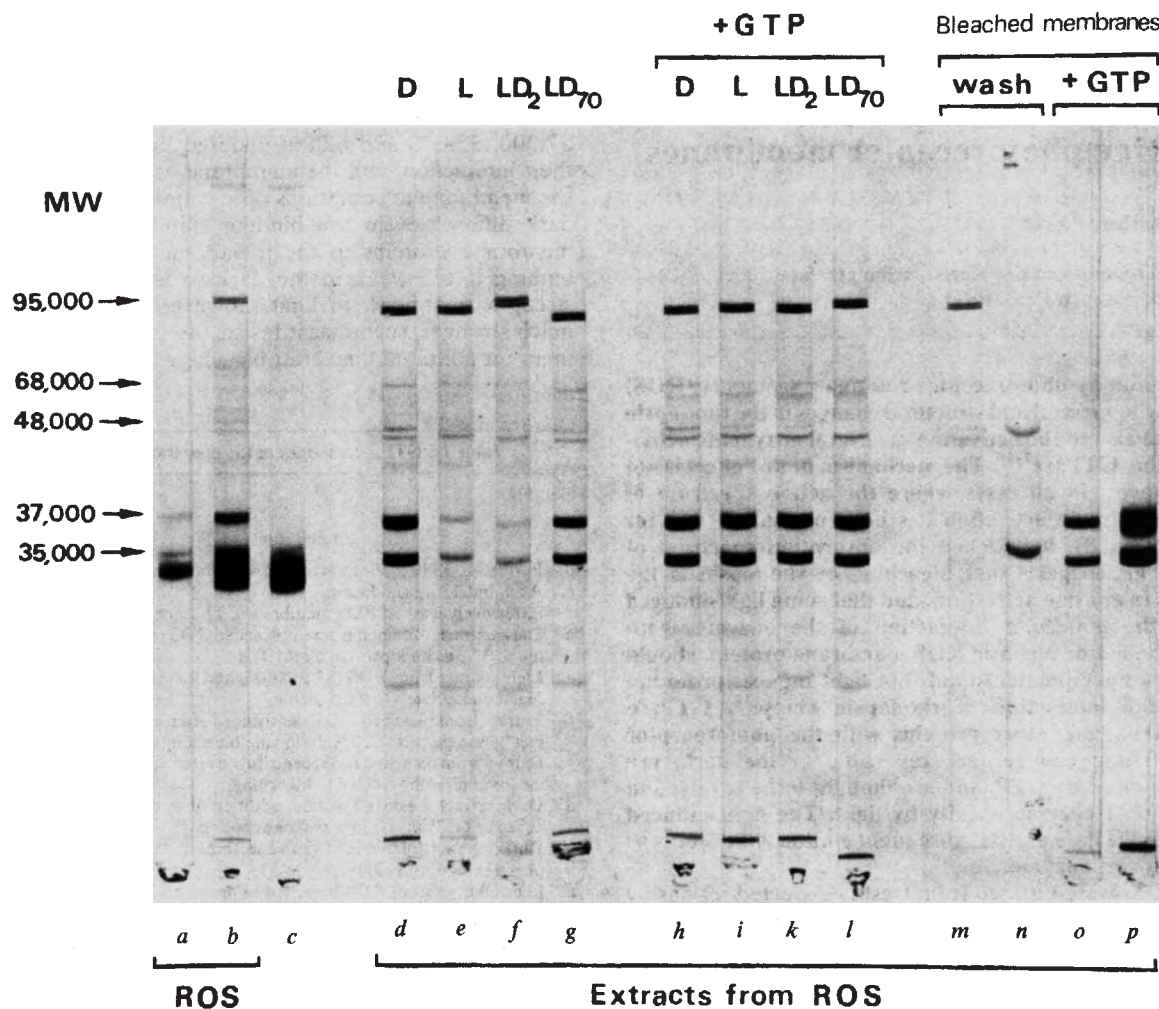


Fig. 1 Polypeptide composition of ROS and extracts, analysed by electrophoresis on discontinuous, 10% polyacrylamide gels in the presence of 0.1% SDS¹⁷. Gels were stained for protein with Coomassie brilliant blue. For definition of the lettered samples see Table 1, where the corresponding GTPase activities are also listed. Briefly: gels *a-c* represent ROS membranes before (*a, b*) and after (*c*) extraction of soluble proteins; and gels *d-p* represent soluble proteins extracted from ROS in various light/dark conditions. The extracts *d-l* were all obtained from the same ROS suspension in 5 mM Tris-HCl (pH 7.4): 2 mM dithiothreitol: 0.5 mM MgCl₂: 0.05 mM phenylmethylsulphonyl fluoride. The amount of extract on each gel (*d-l*) corresponds to 35 µg rhodopsin in the original homogenised ROS suspension. The ROSs were extracted either unbleached (*d, h*) or after illumination (*e-g, i, k, l*). The 'light' suspensions were illuminated for 3 min at 20 °C with light filtered through an orange filter ($\lambda > 540$ nm) and through a water bath, bleaching about 80% of the rhodopsin. Then both light and dark suspensions were centrifuged either immediately (*d, e, h, i*) or after an additional incubation period at 20 °C in the dark (*f, g, k, l*). (The light/dark conditions applied before centrifugation are depicted on top of the gels: D, dark extract; L, light extract; LD₂ and LD₇₀, dark incubation for 2 min or 70 min, respectively, following illumination.) The extracts *h-l* were obtained in the presence of 1 mM GTP. For details see Table 1. After the first centrifugation (10 min at 50,000g and 0 °C), the supernatants were carefully removed and centrifuged again for 40 min at the same speed to insure that all particulate material was sedimented. The extracts were clear and free of rhodopsin. The GTPase was purified as follows: An ROS suspension was bleached and centrifuged. The bleached pellet containing the bound GTPase was washed several times with 5 mM Tris at 0 °C to remove residual soluble proteins (*m*) and was then treated several times with 100 mM Tris for 70 min at 20 °C to allow for the release of light-induced bound proteins (*n*) other than GTPase. The GTPase remains membrane-bound in 100 mM Tris. The GTPase was finally eluted by treating the pellet with 40 µM GTP in 5 mM Tris buffer (*o, p*). All buffers used contained 2 mM dithiothreitol. MWs of polypeptides are indicated on the left side of the gels and were estimated from gels run separately in a continuous electrophoretic system^{15,18}. The most prominent band in ROS, migrating on Laemmli gels¹⁷ below the extractable polypeptide of MW 35,000, is rhodopsin, as identified by glycoprotein staining (not shown). Polypeptides whose MWs are less than 20,000 migrate with the front on these gels¹⁷. This front band contains a light-dependent component which is seen in the extracts *d* and *g-l*, and in the purified GTPase (*o, p*) but is absent in the light extracts *e* and *f*. Analysis in a gel electrophoretic system designed to separate in the MW range of 2,000–20,000 (ref. 19) indicates a MW of about 6,000 for this light-dependent peptide. It always accompanies the GTPase and is, therefore, tentatively regarded as a subunit of the GTPase in addition to the polypeptides of MWs 37,000 and 35,000. The polypeptide of MW 68,000 carries rhodopsin kinase activity¹¹. The polypeptide doublet of MW 95,000, in contrast to the other designated polypeptides, is extracted independently of light.

GTPase activities of the different fractions from Fig. 1 are presented in Table 1. Whole ROS, if assayed in the conditions described by Wheeler and Bitensky⁸, exhibit GTPase activities in the same range as those reported for frog ROS in light⁸. Dark extracted and thoroughly washed ROS membranes (Fig. 1c) contain no GTPase activity (Table 1c). The extracts also show negligible GTPase activity (Table 1d'), unless assayed in the presence of added, GTPase-depleted bleached ROS membranes. Then the dark extracts show high GTPase activities, up to 40 times higher than the corresponding light extracts

(Table 1d, e). Obviously, the GTPase is extracted from dark-adapted membranes in an inactive form and becomes active only when bleached ROS membranes are added. Extracts of bleached ROS, on the other hand, contain little GTPase because of the light-induced, strong binding of the enzyme to the membranes; but after prolonged incubation in the dark, the strongly bound GTPase is reconverted into the form where it can be extracted at low ionic strength (Table 1g). Comparison of GTPase activities and polypeptide composition of many extracts showed that the presence of the three light- and GTP-influenced

polypeptides (MWs 37,000, 35,000 and about 6,000) always and quantitatively paralleled the presence of GTPase activity. This strongly suggests that at least one if not all of these polypeptides constitute the GTPase. This is in essential agreement with recent findings of Godchaux and Zimmerman¹⁶ who also assign GTPase activity to the polypeptide doublet of MWs 37,000 and 35,000 (which they term 41K and 37K).

The data in Table 2 show that light changes the interaction between the GTPase and ROS membranes even in moderate ionic strength conditions where the enzyme is membrane-bound both in darkness and light (extracts D1, L1). Bleaching membranes suspended in 100 mM Tris buffer prevents the enzyme from being extracted in the dark by a subsequent treatment with 5 mM Tris (extract L2), whereas the enzyme from a similarly treated unbleached sample can be extracted in the same conditions (extract D2). It seems likely, therefore, that significant light-induced changes in the binding of the GTPase to ROS membranes occur also in physiological ionic strength conditions.

Table 2 Effect of the ionic strength on the extractability of GTPase from unbleached and bleached ROS

Treatment of ROS suspension before centrifugation	Light conditions	Extract	GTPase activity of extracts
<i>First extraction</i> ROS vigorously homogenised in 100 mM Tris and divided into two samples for dark (D) and light (L) extraction	Dark	D1	1.64
	Light	L1	1.05
<i>Second extraction</i> Pellets resulting from the first extraction resuspended in 5 mM Tris	Dark	D2	20.0
	Dark	L2	0.45

The conditions of illumination and centrifugation were as described in Fig. 1. The time elapsed between the first and second centrifugation step was 15 min during which the membranes were kept in the dark mostly at 0–4 °C. Both buffers contained 0.5 mM MgCl₂, 1 mM dithiothreitol, and 0.05 mM phenylmethylsulphonyl fluoride in addition to the Tris-HCl (pH 7.4; 100 mM or 5 mM, respectively). Neither of the extracting buffers contained GTP. The homogenisation treatment was sufficient to disrupt the ROS structure such that most of the soluble proteins were eluted in 100 mM Tris buffer, except the polypeptides of MWs 95,000 (doublet), 37,000, 35,000 and 6,000 which are membrane-bound at this ionic strength. The latter five polypeptides were found enriched in the subsequent hypotonic extract D2, whereas L2 contained only the doublet of MW 95,000 (gels not shown). GTPase activity was measured and is expressed as for Table 1.

The light-induced binding of the GTPase and its specific elution with GTP offers a unique way of purifying this enzyme. Bleached ROS are washed extensively to remove soluble proteins (Fig. 1e, m, n) and are then incubated with 40 µM GTP in hypotonic buffer. The resulting extract contains high GTPase activity if assayed in the presence of added bleached ROS, and the only polypeptides present are those of MWs 37,000, 35,000, and about 6,000 (Fig. 1o, p; Table 1o). The yield of purified GTPase is 60–70 µg of each of the two large polypeptides (MWs 37,000 and 35,000) per mg of rhodopsin; this indicates that at least 6–7 molecules of each polypeptide are present per 100 rhodopsin molecules in ROS.

The phenomenon of light-induced changes in the interaction of certain ROS proteins with the photoreceptor membrane may well reflect the mechanism by which enzymes in ROS are light-activated via bleaching of rhodopsin. To support this hypothesis, it would be important to know how rapidly the light-induced binding changes occur. Preliminary experiments¹⁵ indicate that, even at 0 °C, both the light-induced binding and the GTP-induced release of the GTPase occurs faster than the time resolution of the centrifugation method (10–15 min). This leaves open the possibility that, at physiological temperatures,

the observed changes in molecular interactions of ROS proteins may be fairly fast reactions.

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Epithelial cells in nerve-free hydra produce morphogenetic substances

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It has been shown that hydra, consisting almost exclusively of epithelial cells, have normal morphogenetic properties. This means that they are able to reproduce asexually by budding and to regenerate head or foot with conservation of the original polarity. Such hydra have no interstitial cells and they lack nerves and nematocytes as differentiation products of the interstitial stem cell. Consequently, they are unable to catch or ingest food and would not survive in nature. However, stocks can be maintained in the laboratory, if food is injected into the gastric cavity^{1–3}. The fact that nerve-free hydra would grow and regenerate normally raised some doubts as to the role of the nervous system in the control of differentiation and development in hydra. In particular, the significance of morphogenetic substances, such as the head activator⁴, which is normally produced by nerve cells⁵, seemed to need reassessment⁶. We now show that epithelial cells in hydra have the potential to produce head activator and other morphogenetic factors, but that this property is repressed in the presence of nerves.

In a preliminary investigation we have shown that severely nerve-depleted hydra, which consist almost exclusively of epithelial cells, contain normal concentrations of morphogenetic substances⁷. To exclude the possibility that the few remaining nerve cells overproduce these substances, we have repeated the experiments with completely nerve-free hydra. These were from two sources: the first 100 were from Richard Campbell from a

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Table 1 Cell composition of nerve-free and normal hydra

Animal	Total cells (%)	Epithelial	Gland and mucous	Interstitial	Nematocyte lineage	Nerve
Nerve-free	14,700 (100)	13,500 (91.8)	660 (4.5)	320 (2.2)	210 (1.4)	0
Normal	62,300 (100)	13,800 (22.0)	3,930 (6.3)	7,100 (11.4)	34,300 (55.0)	3,200 (5.1)

stock originally produced by colchicine treatment and had been nerve free for more than a year⁸. The next 300–400 nerve-free hydra we produced ourselves following C. N. David's suggestion of selecting non-feeding animals from the normal culture and force-feeding them by hand. Such animals are probably the result of interstitial stem-cell depletion caused by an excessive differentiation into nerves or nematocytes or, experimentally induced, into sexual cells. Such animals were not interstitial-cell free, and they contained a few remaining nerve cells, but they produced offspring which in most cases was completely nerve free. Such nerve-free hydra were easily recognisable by their characteristic shape, with many undetached buds, swollen heads and short tentacles (Fig. 1a), by their inability to catch food and by their lack of reaction against tactile stimuli such as tickling the

1 reflects the status of animals that have been nerve free for a few generations. The longer such animals are propagated by force-feeding, the more they lose cell types other than the epithelial cells, such that in the end they consist exclusively of epithelial cells. As Campbell's animals had reached that stage, we are sure that it is the epithelial cell which is producing morphogenetic substances. Campbell and Marcum produced nerve-free hydra by colchicine treatment⁸, and Sugiyama *et al.* selected for an interstitial-cell-free mutant by sexual inbreeding³. The fact that our animals were asexual offspring from normal hydra, not treated with chemicals or mutagens and not the result of sexual crosses, suggests that the ability to produce morphogenetic substances is an inherent property of epithelial cells and not the result of a mutation or chemical mistreatment.

We had shown earlier that in normal animals most of the head activator (80–90%) is located in nerve cells⁵. This did not exclude the possibility that the remaining 10–20% may be present in another cell type. To obtain direct evidence on the occurrence of head activator in normal epithelial cells a procedure was devised for separating nerves from epithelial cells. Hydra were dissociated into viable cells according to the method described in Trenkner *et al.*¹¹. Epithelial cells were separated from nerve cells using sedimentation velocity centrifugation in Percoll gradients. Because the two cell types differ greatly in size (average nerve cell and epithelial cell diameters are 6.0 and

Table 2 Amount of material necessary to achieve the effect corresponding to 1 biological unit (BU)

Morphogen	Nerve-free hydra	Normal hydra	Ratio
Head activator	0.009	0.075	8.3
Head inhibitor	0.009	0.033	3.7
Foot activator	0.05	0.09	1.8
Foot inhibitor	0.14	0.11	0.8

The amount of material is given in A_{280} units per ml. One standard nerve-free hydra, if homogenised in 1 ml, yielded on average 0.14 A_{280} units and one standard normal hydra 0.15 A_{280} units. One biological unit is defined as the amount of material necessary in 10 ml of medium to achieve for the head activator a 6% increase in tentacle number, a 50% increase in bud outgrowth, or a 33% acceleration in head-regeneration rate, for the head inhibitor a 50% decrease in bud outgrowth or a 33% inhibition of head-regeneration rate, for the foot activator a 50% acceleration and for the foot inhibitor a 25% inhibition of the foot-regeneration rate¹⁰.

tentacles with a needle⁹. To standardise size we selected animals in which a first bud was just visible (Fig. 1b). Such standard animals consisted on average of $15,000 \pm 2,000$ cells, of which more than 90% were epithelial cells (Table 1).

From these hydra we made crude extracts and assayed them directly and after purification for content of head activator, head inhibitor, foot activator and foot inhibitor¹⁰. We found that nerve-free hydra contain eight times more head activator, four times more head inhibitor, twice as much foot activator and a little less foot inhibitor than normal animals (Table 2). No difference was found in the head-activator concentration of nerve-free hydra originating from the two sources. From this we conclude that cell types other than the nerve cells can produce morphogenetic substances. The cell composition shown in Table

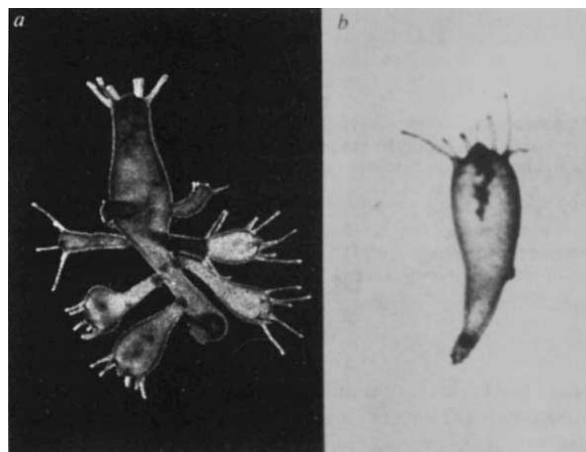


Fig. 1 Nerve-free hydra. *a*, Mother animal with several buds which need much longer to detach than normal buds. Each individual bud has to be force-fed by hand. *b*, Standard animal which started to develop its first bud.

Table 3 Head activator in cell fractions enriched for epithelial or nerve cells

Animal	Cell fraction	Cell composition ($\times 10^6$)			Content of head activator (BU)
		Total	Epithelial	Nerve	
Normal	Unseparated	90.0	20.0	4.5	400
	Epithelial enriched*	9.4	6.0	0.06	35
	Nerve-enriched†	5.3	1.9	2.8	320
Nerve-free	Unseparated	3.8	3.5	0	400

* $2-3 \times 10^6$ cells were layered over an 8-ml linear Percoll gradient with a density range of 1.01–1.02, and separated by centrifugation at 300g for 10 min. Cells in the lower one-third of the gradient plus the pellet were pooled.

† Nerve cells were enriched using glycerol gradients as described earlier^{5,12}.

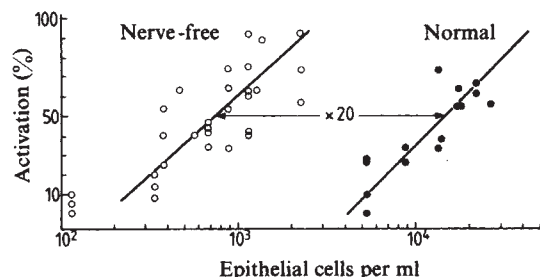


Fig. 2 Head activator in epithelial cells of nerve-free and normal hydra.

15.6 μm , respectively), a fraction could be obtained consisting mostly of epithelial cells and containing very few nerves (Table 3). From an epithelial-cell-enriched fraction a crude extract was prepared, and after purification on G-10, assayed for content of head activator. We found that it contained ~9% of the head activator present in unseparated cells (Table 3). Because only 1% of the nerve cells were represented in this fraction, we conclude that a minor portion of the head activator may be produced in normal animals also, by cell types other than the nerve cells, possibly epithelial cells. Figure 2 compares the content of head activator per epithelial cell in this isolated epithelial cell fraction from normal animals with that in nerve-free hydra. The results show that epithelial cells from nerve-free hydra contain at least 20 times more head activator than epithelial cells of normal animals. This is interpreted to mean that

epithelial cells in normal animals may have the capacity to produce morphogenetic substances, but that this capacity is repressed in the presence of nerve cells.

Thus, our findings indicate that nerve-free hydra contain the same morphogenetic substances as normal hydra. One argument against a morphogenetic role for these substances was that they are neurohormones and are involved in a regulation imposed by the nervous system. The finding that no major difference exists in the morphogenetic properties between normal and nerve-free hydra supports the notion that hydra and morphogenetic substances in hydra are a good model for studying pattern formation in embryological systems.

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Expression of herpesvirus-induced antigens in human cervical cancer

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Herpes simplex virus type 2 (HSV2) was first associated with human cervical cancer by seroepidemiologic studies over 10 years ago, but there has been no clearcut evidence confirming or refuting the role of the virus in this cancer. Expression of HSV antigens in cells transformed *in vitro* by the virus has been reported by several groups^{1,2}. Evidence for the association of HSV antigens with human cervical cancer has recently been reviewed^{3–5}; general antisera prepared against HSV virions or structural proteins were used by several groups to screen cells derived from the cancer and equivocal results were obtained. The tumour-specific antigens associated with three known oncogenic DNA viruses—adenovirus type 2, SV40, and Epstein-Barr herpesvirus—are DNA-binding proteins^{6–8}. Therefore, proteins which bind DNA were isolated^{9,10} from HEP-2 cells infected by HSV2, and monospecific antisera to two of these proteins (ICSP 11/12 and ICSP 34/35) were prepared. These rabbit antisera react specifically with the nuclei of HSV2-infected cells¹¹. We show here that an HSV2-specific DNA-binding antigen was expressed in 38% of tissues with pathological findings of severe dysplasia or carcinoma. This therefore constitutes further evidence for HSV2 as a factor in human cervical carcinoma.

Prepared antisera to purified HSV2 virions (anti-HSV2) anti-ICSP 11/12 and anti-ICSP 34/35, and pre-inoculation rabbit serum were used to screen human cervical tissue for the presence of HSV-specific antigens. The cervical tissues were embedded in OCT compound (Lab-Tek) and quick frozen in a cryostat. Sections (10 μm) were cut for haematoxylin/eosin (HE) staining, and additional sections (6 μm) were cut for immunological staining procedures. All HE stained slides were double-blind coded for pathological examination. Adjacent sections were stained both by indirect immunoperoxidase (IP) staining and by indirect immunofluorescence (IF) staining¹². All of the antisera (primary and goat anti-rabbit horseradish peroxidase or fluorescein conjugates) were adsorbed with uninfected Hep-2 cells and with rabbit liver powder before use.

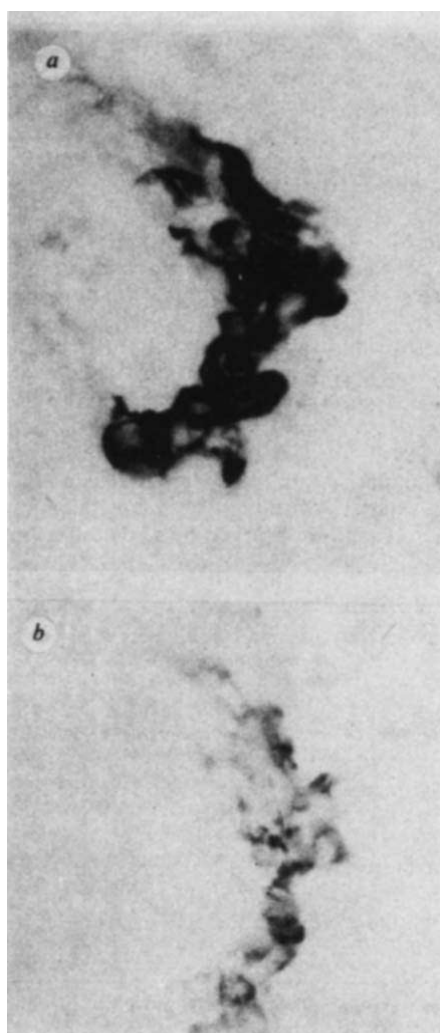
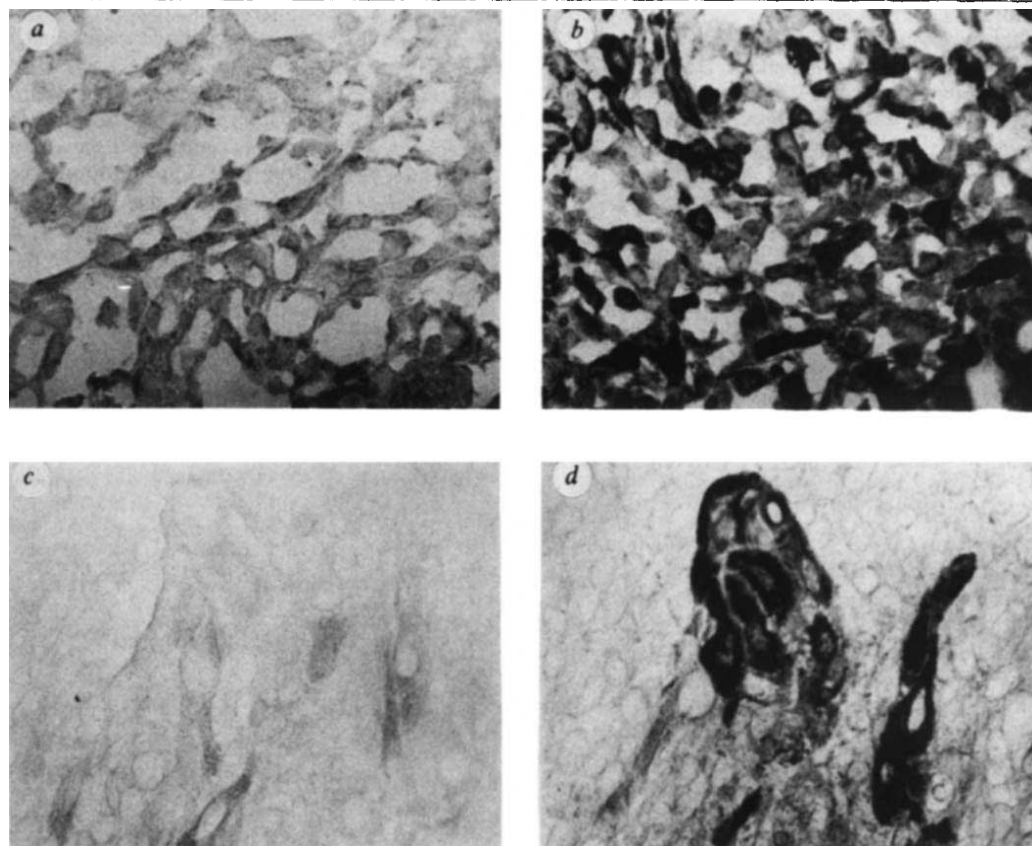
Tissues obtained from 124 women were screened for the presence of HSV2-specific antigens using the above sera. The results are summarised in Table 1. Whereas anti-ICSP 34/35 gave a nuclear pattern of staining with infected Hep-2 cells, all of the positive reactions observed by screening biopsy material

Table 1 Staining of human cervical biopsy sections by indirect immunoperoxidase

Diagnosis	Total tested	No. negative	No. positive	Per cent positive
Normal	15	15	—	0
Chronic inflammation	6	6	—	0
Metaplasia	27	26	1	4
Dysplasia				
Mild	35	34	1	3
Moderate	20	18	2	10
Severe	11	7	4	36
Carcinoma <i>in situ</i> and invasive	10	6	4	40

The sections were stained with rabbit anti-HSV-specific ICSP 34/35 sera.

Fig. 1 Immunoperoxidase staining of human cervical tissues. *a* and *b*, Tissues were from a patient diagnosed as severe dysplasia; *c* and *d*, tissues were taken from a patient with carcinoma *in situ*. The primary antisera used were rabbit anti-ICSP 34/35 (*b* and *d*) and rabbit pre-inoculation (*a* and *c*). $\times 560$.



were perinuclear and cytoplasmic. Typical patterns are shown in Fig. 1. This staining pattern is similar to that observed previously by Flannery *et al.*², who used an antiserum designated anti-VP143 to stain a number of HSV2-transformed cell lines. The VP143 HSV2 protein has the same molecular weight as ICSP 11/12. When the tissues were stained using the IF technique, the results were the same as those given in Table 1. If the tissues are divided into two groups (group I, those with diagnoses of normal, chronic inflammation, metaplasia, mild dysplasia, and moderate dysplasia; and group II, those with severe dysplasia, carcinoma *in situ* and invasive carcinoma), the following observations can be made: the incidence of positives in group I is 4 out of 103, or 4%, and in group II is 8 out of 21, or 38%. Similar but weaker patterns were observed when the majority of the above tissues were studied using anti-ICSP 11/12. However, no reactivity was seen using antiserum to HSV2 whole virions. Comparable results have been obtained by McDougall *et al.* (personal communication) using anti-ICSP 11/12.

The specificity of the reactions has been tested by adsorbing the anti-ICSP 34/35 sera with HSV2-infected HEP-2 cell lysates. The adsorbed sera were now negative when allowed to react with infected cells. The adsorbed sera were also tested with cervical tissue sections from seven positive patients. In six cases the reaction was now negative or significantly diminished (Fig. 2*a, b*). In the seventh case, the reaction was essentially unchanged. The six tissues in which the reaction was blocked or significantly diminished had a pathological reading of severe dysplasia or carcinoma. The seventh tissue, the reactivity of which was not altered by blocking, was a section from tissue diagnosed as metaplasia. The significance of these patterns is being investigated in greater detail.

One tissue with a pathology report of severe dysplasia gave nuclear staining by the IP staining procedure. However, this pattern was also noted when the tissue was reacted with the

Fig. 2 Immunoperoxidase staining of a human cervical tissue with a pathological finding of moderate dysplasia. The primary antiserum used was rabbit anti-ICSP 34/35 before (*a*) and after (*b*) adsorption with HSV2-infected cell lysates. $\times 560$.

anti-HSV2 virion serum, as well as with an anti-HSV1 serum. There was only one localised area in the tissue that contained cells which gave this nuclear pattern of staining.

In summary, an HSV2-specific DNA-binding antigen was expressed in 38% of tissues with pathological findings of either severe dysplasia or carcinoma. This is of additional interest as these same tissues are negative when examined with an anti-virion HSV2 serum. The specificity of the reactivity for HSV DNA-binding protein antigen was demonstrated in that (1) pre-inoculation rabbit serum gave no reaction; (2) 21 tissues with no pathological changes were negative; (3) adsorption of the anti-ICSP 34/35 serum with HSV2-infected cell lysate blocked the reaction; and (4) adsorption with lysates of uninfected HEP-2 cells did not alter the reactivity. Our data lend support to a previous finding that about 60% of human cervical tumours contain HSV mRNA¹³. These fingerprints add further evidence for HSV2 as a factor in human cervical carcinoma.

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(ADP-ribose)_n participates in DNA excision repair

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Chromatin proteins are covalently modified by at least five different processes; in no case has the precise physiological function been established. One of these post-synthetic, covalent modifications is effected by the enzyme poly(ADP-ribose) polymerase, which uses the coenzyme NAD⁺ to ADP-ribosylate chromatin proteins^{1–3}. The modification consists largely of mono(ADP-ribose), but long, homopolymer chains of (ADP-ribose) are also present. Various physiological functions have been suggested for (ADP-ribose)_n. Here we demonstrate that one function of (ADP-ribose)_n is to participate in the cellular recovery from DNA damage. Specific inhibitors of poly(ADP-ribose) polymerase prevent rejoining of DNA strand breaks caused by dimethyl sulphate and cytotoxicity is enhanced thereby. The rejoining of strand breaks is prevented also by nutritionally depleting the cells of NAD.

Both radiation^{4–6} and alkylating agents^{7–10} lower the cellular NAD content. An explanation for this phenomenon was suggested when we showed that the nitrosourea, streptozotocin, both lowered cellular NAD content and raised the specific activity of poly(ADP-ribose) polymerase⁹. Treatment of cells with *N*-methyl-*N*-nitrosourea or with γ -radiation results in a profound drop in cellular NAD content, and this decrease is correlated with enhanced activity of poly(ADP-ribose) polymerase^{10–12}. The enzyme activity also increases when

permeabilised cells are incubated with either endogeneous¹³ or exogeneous endonucleases^{13,14}. Poly(ADP-ribose) polymerase activity increases in human lymphoblastoid cell lines after UV irradiation, and this response is abolished or delayed in xeroderma pigmentosum cells, which are unable to excise pyrimidine dimers¹⁵. Thus DNA fragmentation seems to activate poly(ADP-ribose) polymerase, thereby decreasing the cellular NAD content¹³.

Four chemical classes of inhibitors of poly(ADP-ribose) polymerase are known; nicotinamides (such as 5-methylnicotinamide)^{16–18}, methylxanthines^{10,11,19,20}, thymidine^{16,21} and aromatic amides such as benzamides (refs 21, 22, and G. Khan and S.S. unpublished results). The inhibition constant (*K_i*) for 3-aminobenzamide in isolated L1210 nuclei has been estimated to be $1.8 \pm 0.2 \mu\text{M}$ (M. Purnell and W. J. D. Whish, personal communication). We have confirmed that 3-aminobenzamide is a very powerful, competitive inhibitor of poly(ADP-ribose) polymerase. The *K_i* in permeabilised¹³ L1210 mouse leukaemic lymphoblast cells, at 26 °C is $4.4 \pm 1.1 \mu\text{M}$. This enzyme inhibitor is non-toxic to these L1210 cells at up to 3 mM, as shown by a cloning assay in soft agar. Two enzymes catabolise NAD in eukaryotes—NAD glycohydrolase (EC 3.2.2.6) and poly(ADP-ribose) polymerase. 3-Aminobenzamide does not inhibit NAD glycohydrolase in our system. As 3-aminobenzamide does not reduce the cloning efficiency of these cells, it is likely that it does not have profound metabolic effects of an unknown character in undamaged cells.

The cellular NAD content is lowered by dimethyl sulphate (DMS) in a dose-dependent manner (Fig. 1a), reaching the minimum level in 1 to 2 h of treatment. Poly(ADP-ribose) polymerase activity in nucleotide-permeable cells¹³ after DMS treatment increases (threefold at 1 mM DMS) in a dose-dependent way and shows a peak of activity when the NAD content is dropping most rapidly (Fig. 1c).

At 1 mM, 3-aminobenzamide prevents the NAD decrease completely, while the non-inhibitor, 3-aminobenzoic acid, has no effect (Fig. 1b). The decrease in NAD induced by DMS is also inhibited by 5-methylnicotinamide, thymidine/deoxycytidine and theophylline, but not by nicotinic acid (data not shown). Neither 5-methylnicotinamide nor thymidine/deoxycytidine significantly inhibit NAD glycohydrolase¹². The decrease in NAD content induced by DNA-damaging agents is mediated by poly(ADP-ribose) polymerase; thus (ADP-ribose)_n may be implicated in some aspect of DNA repair.

We have therefore examined the kinetics of removal of single-strand breaks (this includes breaks produced by the alkaline conditions of the gradient) following treatment with DMS. Immediately after treatment, the DNA sediments as a broad peak in the middle of the gradient (Fig. 2c, a). At 40 min after removal of the DMS, the DNA is increased significantly in size (Fig. 2c), and by 80 min it is completely converted into a fast sedimenting complex near the bottom of the gradient, which is the position at which undamaged DNA sediments (Fig. 2d).

However, when the enzyme inhibitor 3-aminobenzamide (3 mM) is added to the cells following removal of DMS, conversion of the low molecular weight DNA to a fast-sedimenting material is almost completely inhibited (Fig. 2a, b). There is no observable increase in molecular weight of the DNA 40 min after removal of DMS (Fig. 2a). After 80 min, the DNA is increased slightly in size, but still sediments in the middle of the gradient (Fig. 2b). Even after 5 h the bulk of the DNA has not yet returned to its original size (Fig. 2b). Addition of 3 mM 3-aminobenzoic acid does not retard excision repair following DMS treatment (data not shown).

Other inhibitors of the polymerase also inhibit excision repair in these cells. Both 5-methylnicotinamide and thymidine (in the presence of deoxycytidine) are effective inhibitors of excision repair (data not shown). Thus we have demonstrated that inhibitors of the enzyme, poly(ADP-ribose) polymerase, inhibit excision repair in these cells.

If (ADP-ribose)_n biosynthesis participates in the cellular recovery from DNA damage, then inhibition of (ADP-ribose)_n

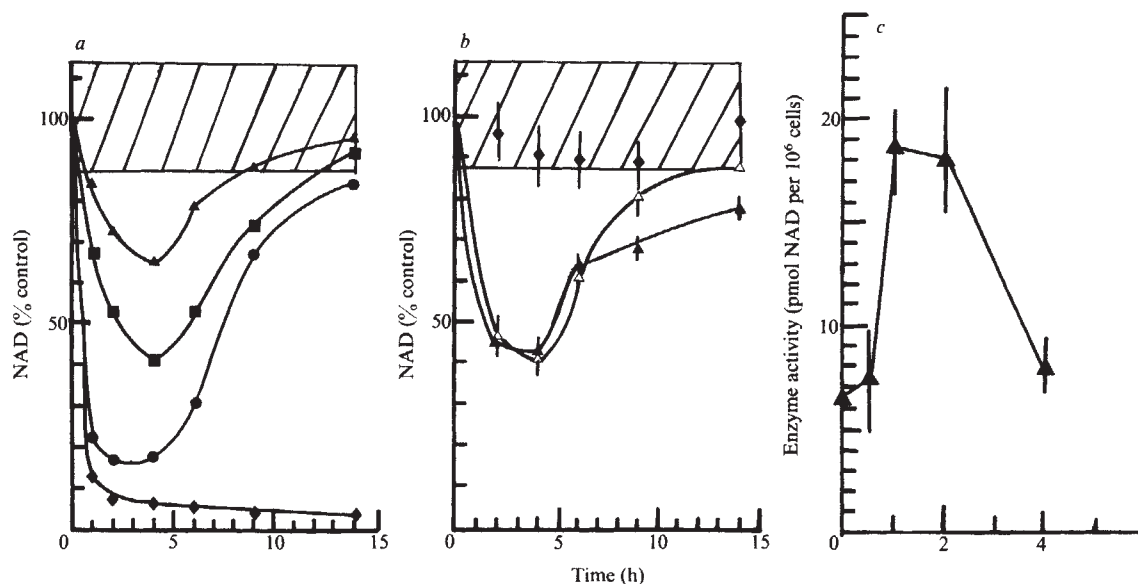


Fig. 1 The effect of DMS and of poly(ADP-ribose) polymerase inhibitors on the NAD content of L1210 cells. *a*, Cells were continuously treated with increasing doses of DMS, and samples were removed at intervals for NAD assay, washed once with 0.9% (w/v) NaCl, resuspended in cold 50% (v/v) ethanol, and then sonicated for 20 s (medium power, amplitude 1, MSE sonicator). Each sample was assayed for NAD in duplicate by a modification of the method of Nisselbaum and Green¹². Δ , 10.5 μ M DMS; $\blacksquare$, 52.6 μ M; $\bullet$, 105.3 μ M; $\blacklozenge$, 210.6 μ M. *b*, L1210 cells were continuously treated with 53 μ M DMS either alone (Δ) or in the continuous presence of either 2 mM 3-aminobenzamide ($\blacklozenge$) or of 2 mM 3-aminobenzoic acid ($\blacktriangle$). Samples were removed at intervals for NAD estimation¹². *c*, Cells were treated continuously with 1 mM DMS and then the poly(ADP-ribose) polymerase activity was determined at the indicated times. Permeabilised cells¹³ were cooled to 4 °C, collected by centrifugation in the cold and washed once with cold Pucks saline (containing 400 mg l⁻¹ KH₂PO₄, 350 mg l⁻¹ NaHCO₃, 8 mg l⁻¹ NaCl, and 1 g l⁻¹ glucose at pH 7.4). The cells were resuspended in a cold hypotonic buffer pH 7.8, (containing 9 mM HEPES, 4.5% (w/v) dextran, 1 mM EGTA, 4.5 mM MgCl₂, and 5 mM DTT). The cell concentration in this hypotonic buffer was $3\text{--}5 \times 10^7$ per ml. After 30 min incubation at 4 °C the cells were diluted 10-fold with an isotonic buffer pH 7.8 (containing 40 mM HEPES, 130 mM KCl, 4% (w/v) dextran, 225 mM sucrose, 2 mM EGTA, 2.3 mM MgCl₂ and 2.5 mM DTT). 250 μ l of cells were incubated at 25 °C with 5 μ l of ³H-NAD (final concentration 2 μ Ci ml⁻¹, 100 μ M). Triplicate samples were taken at 2-min intervals up to 8 min. The reaction was stopped with 4 ml of 20% (w/v) trichloroacetic acid (TCA). The samples were left at 4 °C for 4 h, filtered on to glass fibre disks and washed four times with 10% (w/v) TCA, and twice with acetone. The filters were dried and the radioactivity estimated in a toluene-PP0 scintillant in a Beckman LS233 liquid scintillation counter. Enzyme activity was estimated by least squares regression analysis. The control level of poly(ADP-ribose) polymerase in these conditions was 6.63 ± 0.94 (s.d.) pmol min⁻¹ per 10⁶ cells (nine determinations).

biosynthesis would be expected to increase the toxicity (and perhaps also the mutagenicity) of DNA-damaging agents.

Exposure of L1210 cells to DMS for 1 h results in a dose-dependent cytotoxicity (Fig. 3). 90 μ M DMS yields a 10% survival. However, the toxicity of DMS is greatly increased when 2 mM 3-aminobenzamide is present during both exposure of the cells to DMS and in the subsequent cloning assay. In these conditions 22 μ M DMS is sufficient to give 10% survival; this represents a fourfold dose-enhancement. 3-Aminobenzoic acid (2 mM) has no effect on the cytotoxicity of DMS (Fig. 3). In addition, 5-methylnicotinamide, thymidine/deoxycytidine and theobromine potentiate the cytotoxicity of DMS (O.O. and S.S., manuscript in preparation).

This potentiation of cytotoxicity is observed also with several other monofunctional alkylating agents, and with representatives of each class of enzyme inhibitor (N. Nduka, O.O., A. A. Zia'ee and S.S., manuscripts in preparation).

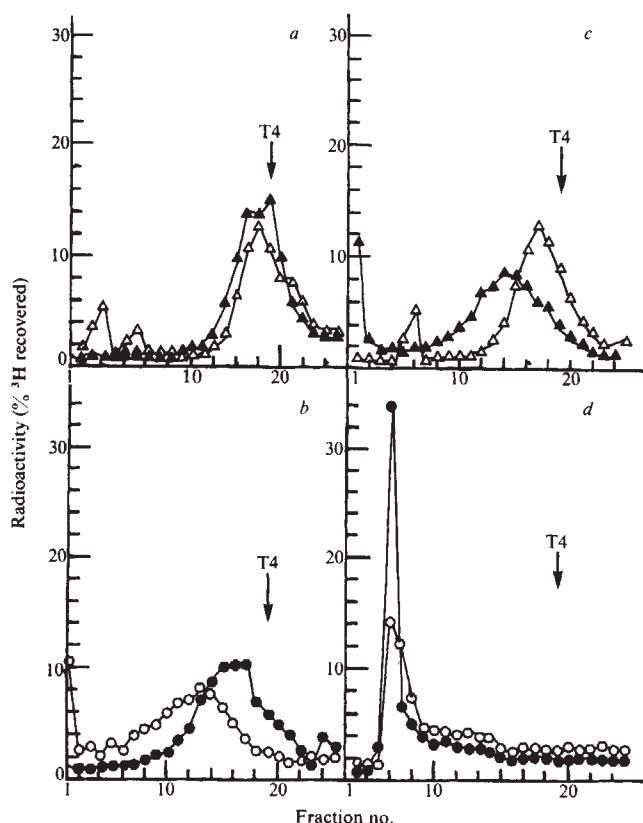


Fig. 2 The effect of 3-aminobenzamide on excision repair in L1210 cells. L1210 cells were prelabelled for 18 h (1.5 generations) with 1 μ Ci ml⁻¹ ³H-thymidine, 1 μ M total thymidine. The radioactivity was washed out and the cells were treated with 100 μ M DMS for 20 min. The cells were collected and resuspended in RPMI 1640 medium and incubated at 37 °C. Samples of 1 ml were removed at intervals, centrifuged and resuspended in cold phosphate-buffered saline and kept on ice. Samples of 25 μ l ($1\text{--}2 \times 10^4$ cells) were taken for each time point and layered on a lysis layer (100 μ l 2% (w/v) SDS, 10 mM EDTA) over a 4.8 ml to 20% (w/v) sucrose gradient (100 mM NaCl, 100 mM NaOH). The gradients were centrifuged for 1 h at 20 °C in a SW 50.1 Beckman rotor at 25,000 r.p.m. Fractions (12 drops) were collected on filter strips and the TCA-insoluble radioactivity was estimated. Time after removal of DMS: $\blacksquare$, 0 min; $\square$, 40 min; $\circ$, 80 min; $\bullet$, 300 min. The total radioactivity loaded onto the gradients was $\sim 2 \times 10^4$ c.p.m.; recovery was between 80 and 100%. *a*, The cells were exposed to DMS for 20 min, which was washed out, but 3 mM 3-aminobenzamide was added to the medium for the 300 min recovery period. *b*, The cells were exposed only to DMS. The arrow shows the position of a ¹⁴C-labelled bacteriophage T4 DNA, single-stranded of molecular weight 55×10^6 .

The evidence that (ADP-ribose)_n biosynthesis participates in cellular recovery from DNA damage rests heavily on experiments with enzyme inhibitors. Although we show that all four classes of polymerase inhibitors are effective, the possibility remains that all these polymerase inhibitors might also coincidentally inhibit some aspect of DNA repair directly. Therefore, we have tested the ability of cells to carry out excision repair when the cellular NAD content is lowered by nutritional deprivation. NAD levels of cells may be depleted by growing them in a nicotinamide-free medium²³. Even with quite low NAD content (10–20% of normal) the L1210 cells continue to grow and divide at the normal rate. The rate of incorporation of ³H-thymidine by these NAD-depleted cells is the same as that of cells grown either in complete medium or in depleted medium supplemented with nicotinamide for 4 h. The NAD content of L1210 cells is 750 pmol per 10⁶ cells; the NAD concentration is therefore about 1,000 μM. The *K_m* value for the DNAase-activated²⁴ polymerase in permeabilised cells¹³ is 328 ± 127 μM. Thus, lowering of the NAD content should decrease the activity of the enzyme, and therefore inhibit DNA repair.

After exposure to 100 μM DMS, the DNA in NAD-depleted cells becomes fragmented (Fig. 4a), and not only is strand-rejoining inhibited, but the DNA is further decreased in size during the subsequent 80 min (Fig. 4a). This inhibition of recovery is alleviated if the cells are pre-incubated for 4 h in the presence of nicotinamide before DMS treatment (Fig. 4b).

NAD-depleted cells in the presence of hydroxyurea do not display unscheduled DNA synthesis after treatment with methylnitrosoguanidine³².

The evidence that (ADP-ribose)_n is involved in DNA repair can be summarised as follows. Both alkylating reagents and radiation lower cellular NAD contents, and they both elevate poly(ADP-ribose) polymerase activity. The drop in NAD content and the rejoining of strand breaks after treatment with

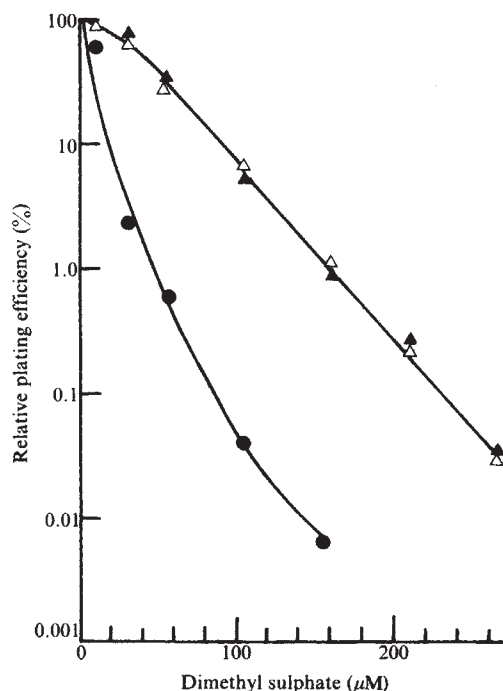


Fig. 3 The effect of 3-aminobenzamide on the cytotoxicity of DMS towards L1210 mouse leukaemia lymphoblast cells. Cells were treated with increasing doses of DMS alone for 1 h at 37 °C (Δ), or with DMS for 1 h in the continuous presence of either 2 mM 3-aminobenzamide (●) or 2 mM 3-aminobenzoic acid (▲). Cells were plated in 0.5% (w/v) agar in RPMI 1640 medium supplemented with 20% (v/v) horse serum in the absence or presence of the inhibitors, respectively. For DMS alone, the points are the means of nine experiments, each with five plates per point; for the other two situations the points are the means of two experiments each with five plates per point.

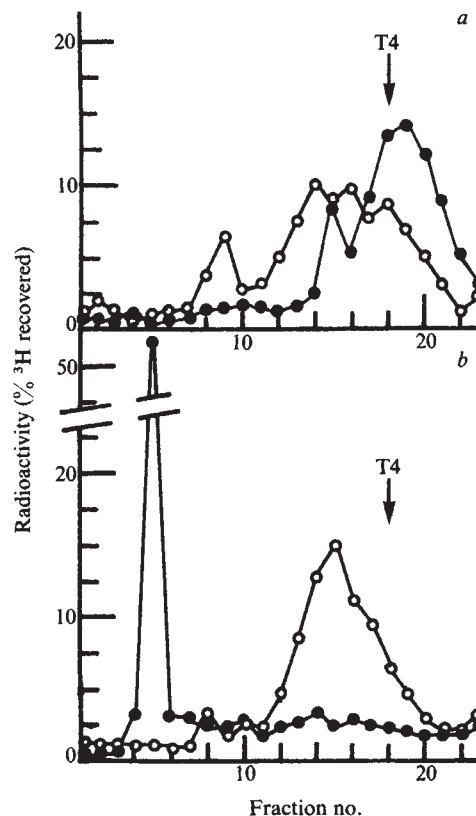


Fig. 4 The effect of NAD depletion on excision repair. Cells were grown for four generations in medium from which nicotinamide was omitted, and the serum was extensively dialysed against phosphate-buffered saline. NAD assays showed that the NAD content was 10 to 20% of the control levels. The culture was divided into two aliquots and nicotinamide was added to one to a concentration of 9 μg ml⁻¹. The cells were exposed 4 h later to 100 μM DMS for 20 min and then their ability to carry out excision repair was tested as described in the legend to Fig. 2. Time: ○, 0 min; ●, 80 min. a, NAD-depleted cells. b, Nicotinamide added back; the NAD level had returned to normal.

DMS are inhibited by 3-aminobenzamide and the other poly(ADP-ribose) polymerase inhibitors, all of which potentiate the cytotoxicity of the alkylating agent. In addition, cells that are nutritionally deprived of NAD cannot efficiently execute excision repair. *N*-methyl-*N*-nitro-*N*-nitrosoguanidine lowers cellular NAD levels and increases intracellular poly(ADP-ribose) levels³³.

The mechanism involved when (ADP-ribose)_n participates in the cellular recovery from DNA damage is not yet understood. Two general types of mechanism may be considered. A protein (enzyme) specifically involved in the molecular DNA repair mechanism may be activated or inhibited by ADP-ribosylation. For example, ADP-ribosylation of a Ca²⁺, Mg²⁺-dependent endonuclease results in the reversible inhibition of nuclease activity²⁵. Alternatively, ADP-ribosylation of either a specific or a nonspecific set of chromatin proteins would profoundly alter local chromatin structure. It has been suggested that (ADP-ribose)_n may have cross-linking properties, or may be involved in extending and condensing chromatin^{26–30}. Cell extracts from some complementation groups of xeroderma pigmentosum can excise pyrimidine dimers from naked DNA but not from chromatin³¹; this implies a need for chromatin structural alterations in excision repair.

Our results show that (ADP-ribose)_n biosynthesis is required for efficient excision repair and survival, following damage with monofunctional alkylating agents. The dramatic synergistic potentiation of cell killing by alkylating agents and poly(ADP-ribose) polymerase inhibitors may be of use in the treatment of human leukaemia.

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Mutagenicity of carcinogenic methylating agents is associated with a specific DNA modification

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The carcinogenic potency of simple aliphatic alkylating agents such as the alkyl nitrosamides and the alkylmethanesulphonates is positively correlated with their ability to alkylate the relatively weakly nucleophilic oxygen atoms in DNA, particularly the O^6 -atom of guanine^{1–4}. Differences in the spectrum of DNA alkylations produced by these agents can be rationalised on chemical grounds in that the electrophilic reactivity of the alkylating species determines the extent to which it will react at sites of weaker nucleophilicity^{4–6}. Alkylation of the more strongly nucleophilic ring nitrogen atoms of the purine bases, which is the main site of reaction with all these agents, appears to be much less important in alkylation carcinogenesis. O^6 -alkylation of guanine is likely to interfere with DNA base-pair hydrogen bonding⁷ and is possibly the major DNA modification responsible for the induction of GC → AT transition mutations in bacteria and bacteriophage by alkylating agents^{8–10}. Here, we have studied the effects of three methylating agents of contrasting carcinogenic potency on mammalian (V79 Chinese hamster) cells in culture. We report that the mutagenicity but not the cytotoxicity of each agent reflects its carcinogenicity and, furthermore, that the marked differences in mutagenicity are closely paralleled by differences in levels of O^6 -guanine methylation.

The somatic mutation theory of cancer requires that all carcinogens be mutagens and that there be some quantitative

relationship between their carcinogenicity and mutagenicity. To test this prediction, we have developed reliable and efficient methods for measuring carcinogen-induced gene mutation frequencies in cultured mammalian cells, based on classical drug-resistance markers, along with techniques for the isolation and chemical analysis of carcinogen-modified DNA. We have previously used the induction of 8-azaguanine-resistant (azg^r) and ouabain-resistant (oua^r) V79 Chinese hamster cells to define the relationship between carcinogenicity, mutagenicity and DNA reaction for various polycyclic hydrocarbon carcinogens^{11–14}. In particular, *anti*-benzo(a)pyrene-7,8-dihydrodiol-9,10-oxide, the proposed ultimate carcinogenic metabolite of benzo(a)pyrene, was an exceptionally potent mutagen in this system¹². Since V79 cells do not excise O^6 -methylguanine from their DNA (ref. 15), they are also ideal for use in experiments designed to establish the role of this base in the mutagenicity and cytotoxicity of methylating agents. For the present investigation, we selected three methylating agents: dimethylsulphate (DMS) and methylmethanesulphonate (MMS) both relatively weak carcinogens and *N*-methyl-*N*-nitrosourea (MNUA), a highly potent carcinogen¹⁶. Quantitation of cytotoxicity and mutation induced by these compounds, coupled with high-pressure liquid chromatography of hydrolysed DNA from cells treated with radiolabelled MNUA and DMS, has enabled the modification specifically responsible for their mutagenic effects to be identified.

The results of several experiments in which the mutagenic effects of DMS, MMS and MNUA were compared in identical experimental conditions are summarised in Fig. 1. Parallel cultures of exponentially dividing V79 cells were exposed for 3 h to a range of concentrations of each compound, and replicate samples were plated for determination of induced cytotoxicity and frequency of azg^r and oua^r mutants; the experimental details are given in the legend to Fig. 1. With all three compounds a similar type of dose-survival curve was obtained, comprising a shouldered (quasi-threshold) region at low doses followed by a steeper portion where survival dropped rapidly with increasing dose. However, the dose range required to achieve this response varied approximately in the ratio 1:3:6 for DMS, MMS and MNUA respectively, the corresponding D_{37} values being 0.11 mM, 0.32 mM and 0.73 mM. The mutational response did not reflect the differences observed between the compounds in survival experiments; MNUA was by far the most potent agent at inducing both azg^r and oua^r clones. With all three compounds, dose-response curves for the induction of oua^r mutants were linear (above 10% survival where mutation frequencies could be measured most accurately) while those for azg^r mutants were cumulative. The contrasting mutagenicity of the agents was more clearly illustrated if induced mutation frequencies were plotted as a function of the logarithm of the corresponding percentage survival, as in Fig. 1. These plots were linear, as routinely found with other classes of mutagen^{14,17}; lines of best fit were determined by least-squares linear regression analysis. The ratio of the slopes of these lines (mutagenic efficiency^{14,18}, or mutagenicity per lethal event) of MNUA relative to DMS, was approximately 15:1 for azg^r mutants and 9:1 for oua^r mutants. Although MMS was less potent than DMS as a mutagen in terms of applied dose (mutagenic effectiveness^{14,18}) the two agents were indistinguishable when their mutagenicity was expressed as a function of induced cytotoxicity. This is explained by the fact that the rate of reaction of DMS is faster than that of MMS (half-time for reaction with DNA *in vitro* is 16 min compared with 5 h) although both agents share similar reaction mechanisms and produce similar proportions of the various methylated DNA products^{4,19}. To confirm that only true mutants were scored in this series of experiments, mutant clones were isolated at random from plates and the cells grown in mass culture. All clones isolated remained fully resistant to the drug used to select them even when propagated for many generations in its absence. In addition, cells possessing the azg^r phenotype invariably had markedly reduced or undetectable amounts of the purine salvage enzyme hypoxanthine-guanine phospho-

Table 1 Extent of methylation of V79 cell DNA at major sites following exposure of cells in culture for 3 h to ^{14}C -DMS and ^{14}C -MNUA

Compound	Survival (%)	Biological effects	Extent of methylation of DNA (μmol per mol DNA P)		
		azg ^r mutation frequency per 10^5 survivors	7-methylguanine	3-methyladenine	O ⁶ -methylguanine
DMS ($8\mu\text{g ml}^{-1}$)	82	10	48.1	8.8	ND
DMS ($15\mu\text{g ml}^{-1}$)	58	34	92.4	12.0	0.5
MNUA ($30\mu\text{g ml}^{-1}$)	81	148	50.3	5.4	7.5
MNUA ($60\mu\text{g ml}^{-1}$)	48	373	102.0	6.2	14.4

In these experiments, each 175 cm^2 plastic tissue culture flask (Nunc UK) was inoculated with 10^7 exponentially growing V79/4-K₁ cells in 50 ml DMEM. After incubation at 37°C for 24 h, groups of five such cultures (about 2×10^8 cells) were treated either with ^{14}C -MNUA or ^{14}C -DMS (Radiochemical Centre, Amersham) of specific radioactivity 11 mCi mmol^{-1} and 18 mCi mmol^{-1} respectively. These doses were chosen to give approximately 80% or 50% survival (see Fig. 1). Immediately before treatment, stocks of the compounds were prepared at 250 times the required treatment concentration in DMSO which had been dried over Molecular Sieves (Fisons). Control cultures were treated with 0.2 ml DMSO alone. After 3 h exposure to the compounds the cells were detached from the flasks with trypsin/EDTA and samples taken for assay of survival and mutation frequency as described in the legend to Fig. 1. The remaining cells were washed twice in phosphate-buffered saline by centrifugation and resuspension. DNA was isolated from cell pellets by a modification of the method of Kirby²⁴. Prior to chromatography, DNA samples were dissolved in 0.02 M phosphate buffer and hydrolysed in 0.1 M HCl at 70°C for 30 min to liberate normal and methylated purine bases. The products of hydrolysis were chromatographed, together with marker alkylated bases, using a Waters ALC GPC 204 liquid chromatograph equipped with a Model U6K injector, a Model 440 ultraviolet detector (Waters Associates) and two columns ($25\text{ cm} \times 0.9\text{ cm}$) of Partisil-10 SCX cation exchanger (Whatman) connected in series with a precolumn of the same material. Separation of methylated purine bases was achieved by elution for 40 min (4 ml min^{-1}) with a gradient running from 0.02 M ammonium formate to 0.2 M ammonium formate, pH 4.0, in 8% (vol/vol) methanol²¹. Values for the extent of methylation at various DNA sites (expressed as μmol per mol DNA P) were calculated from the radioactivity associated with the respective peaks and the specific radioactivity of the methylating species. The amount of DNA analysed was calculated from the ultraviolet absorbance of column fractions containing adenine and guanine. The resolution of O⁶-MeGua was improved if the fractions containing this base were pooled, rotary evaporated at pH 8.0 and rechromatographed on an ODS μ -Bondapak column eluted with a gradient running from 5% methanol: 95% 0.05 M ammonium formate (pH 4.6) to 55% methanol: 45% 0.05 M ammonium formate. ND = not detectable.

ribosyl transferase (HPRT; EC 2.4.2.8)²⁰. These results will be reported fully elsewhere.

In order to investigate observed differences in mutagenic efficiency at the DNA level, the scale of experiments was increased to permit sufficient quantities of carcinogen-modified V79 cell DNA to be isolated and analysed. Cells were exposed to ^{14}C -MNUA and ^{14}C -DMS labelled in the methyl groups. Doses of each compound were chosen with the aim of reducing cell survival to either 80% or 50% of the control plating efficiency. After sampling cells for analysis of survival and mutation frequency, DNA was isolated, hydrolysed and methylated products separated by high-pressure liquid chromatography as described previously²¹. Table 1 summarises the results of typical experiments. At approximately equal levels of cytotoxicity the overall levels of DNA methylation were similar for DMS and MNUA and were proportional to the dose applied. Mutation frequencies (azg^r) at a given dose were typical of those found in the initial studies with unlabelled compounds. As expected⁴, chromatography revealed three major methylated DNA products: 7-methylguanine (7-MeGua) 3-methyladenine (3-MeAde) and, in the case of MNUA, O⁶-methylguanine (O⁶-MeGua). With DMS, the latter product was detectable only with the more toxic treatment ($15\mu\text{g ml}^{-1}$; 58% survival) and then was only present at one-thirtieth the level found with an equitoxic dose of MNUA. This was the only difference observed between the compounds which could explain their contrasting mutagenic potency. The ratios of the various products did not vary significantly from those previously reported for DNA which had been exposed to the same agents *in vitro* or *in vivo*^{2,4,19}.

A comparison of MNUA- and DMS-induced minor alkylation products, which were not readily detectable at the relatively low extents of methylation where mutagenesis could be measured accurately, was investigated by exposing calf thymus DNA (Sigma) *in vitro* to ^{14}C -labelled compounds. Quantities of highly methylated DNA were obtained from these experiments which permitted more sensitive analysis of alkylation products to be carried out. The results of the analysis are shown in Table 2. The relative proportions of the three major products (7-MeGua, 3-MeAde and O⁶-MeGua) were similar to those determined in V79 cells (Table 1) which confirmed that the reaction of these agents with cellular DNA can be represented, both qualitatively and quantitatively, by such a model system. Moreover, with MNUA, there was again no evidence of any product other than O⁶-MeGua which could account for its potent mutagenic activity.

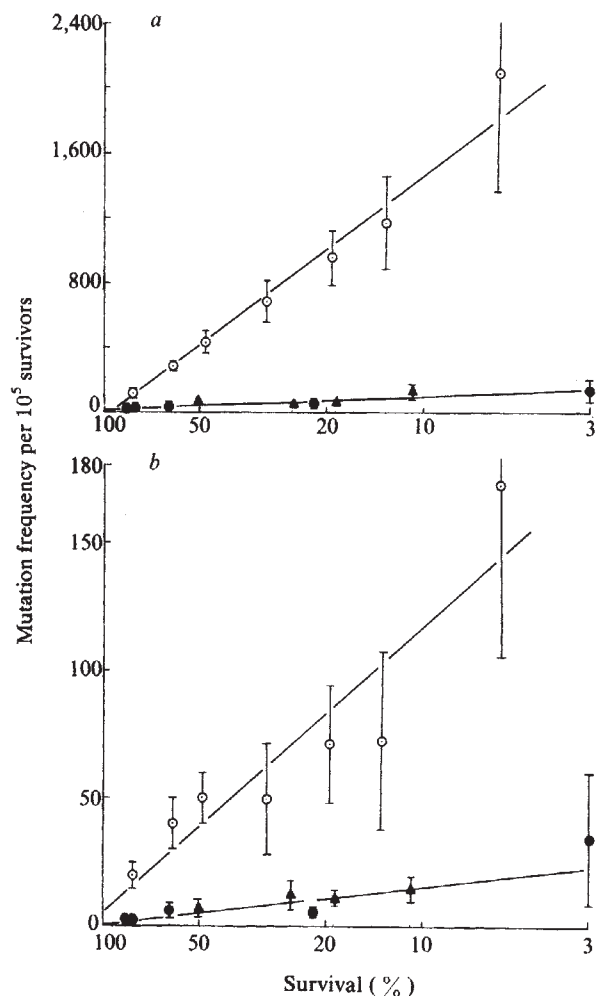
We were not surprised by the lack of a perfect quantitative relationship between the extent of O⁶-guanine methylation and the number of mutants induced by DMS and MNUA. Such a correlation would only be expected if, as seems unlikely, all methylation products other than the O⁶-MeGua (that is the majority of DNA modifications) totally lacked mutagenic effect. Recent studies in this laboratory (J. R. Connell and A.S.C.M., unpublished results) indicate that most chromosomal aberrations induced in V79 cells by these agents are not due to O⁶-MeGua, since similar numbers of aberrations are encountered at equivalent levels of total DNA methylation and cytotoxicity. Although gross damage of this type would not be expected to be particularly efficient at inducing viable azg^r and oua^r mutants in comparison with a miscoding base, it could make

Table 2 Extent of methylation of calf thymus DNA at various sites following treatment *in vitro* with ^{14}C -DMS and ^{14}C -MNUA

Compound	Methylated DNA products (% of total)							
	3-MeGua	7-MeGua	O ⁶ -MeGua	1-MeAde	3-MeAde	7-MeAde	O ² -MeCyt	Apurinic acid
DMS (expt 1)	1.3	70.9	0.3	1.0	14.6	2.0	ND	9.8
DMS (expt 2)	1.2	74.5	0.4	1.1	15.2	1.5	ND	6.1
MNUA (expt 1)	1.0	70.0	7.7	0.7	8.0	0.8	0.1	12.0
MNUA (expt 2)	1.0	64.8	7.2	0.7	9.5	1.4	0.1	15.2

Solutions of calf thymus DNA (5 mg DNA ml^{-1}) in 0.02 M sodium cacodylate buffer (pH 6.9) were treated with ^{14}C -DMS ($6\mu\text{Ci ml}^{-1}$; 60 mCi mmol^{-1}) or ^{14}C -MNUA ($1.8\mu\text{Ci ml}^{-1}$; 11 mCi mmol^{-1}) for 1 h at 37°C with frequent shaking. Immediately following treatment, the DNA was precipitated by the addition of 1/8 vol. 2.5 M sodium acetate and $1\frac{1}{2}$ vols 2-ethoxyethanol. Hydrolysis and chromatography of the DNA were performed as described for Table 1. The values shown are the means of three separate determinations and the extent of methylation of various DNA sites is expressed as a percentage of total DNA methylation.

Fig. 1 Mutagenicity of DMS (●), MNUA (○) and MMS (▲) in V79/4-K₁ cells plotted as a function of percentage survival; *a*, *azg*^r, *b*, *oua*^r. The data, derived from several individual dose-response experiments, were expressed in this way to facilitate direct comparison between the agents in terms of their mutagenic potency per lethal event (mutagenic efficiency^{14,18}). The advantages of this cell line for studies of mammalian cell mutagenesis have been discussed previously¹¹⁻¹⁴. A clone of V79/4 designated V79/4-K₁, selected for its high plating efficiency and its ability to form diffuse microcolonies, was used throughout the course of this work. The latter characteristic allows the efficient recovery of mutants *in situ* which obviates the need to replate cells following each mutation expression period. In some experiments, both methods of selection were used and, invariably the highest mutant frequencies were observed with the *in situ* technique. In all experiments, 75 cm² plastic cell culture flasks (Nunc UK) containing 30 ml of Dulbecco's modified minimal essential medium (DMEM) with 10% fetal calf serum (Gibco) were each seeded with 2×10^6 cells grown from a clonal stock frozen in liquid nitrogen. After incubation at 37°C for 24 h the resulting exponentially growing cultures were treated with various doses of the compounds added in 0.1 ml dry dimethylsulphoxide (DMSO). Control cultures were treated with 0.1 ml DMSO alone. After treatment at 37°C for 3 h the cells were detached from each flask with trypsin-EDTA and the density of the resulting single-cell suspension estimated with a haemocytometer. For the determination of induced toxicity, 2×10^2 cells, and for mutation frequency, 5×10^4 cells, were plated in 5 cm and 9 cm plastic tissue culture dishes (Sterilin) respectively and incubated at 37°C in a humidified atmosphere of 10% CO₂:90% air. Selection of *azg*^r (HPRT deficient) or *oua*^r (ATPase modified) mutants was effected by adding 8-azaguanine or ouabain to the plates such that the final concentration of the drug, in the medium, was 0.2 mM or 1 mM respectively. To achieve maximum recovery of mutants, a complete expression time curve was constructed for each dose of methylating agent. In practice, 5-10 replicates were treated with the selective agent at each of five expression time points ranging from 0-120 h after plating. Survival plates were stained after 7 d of incubation, mutation plates 3 d later, and colonies of more than about 50 cells, with observable mitotic figures, were scored. The plating efficiency of the cells was taken into account when calculating mutation frequencies. The values shown in the figure are derived from at least two separate experiments and are those obtained at peak expression times which ranged from about 48 h for minimally lethal doses to 96 h for the more toxic treatments. However, in all cases, maximum frequencies were observed after the cells had undergone an average of 4-5 divisions. Control frequencies did not exceed 4×10^{-5} per survivor. Standard deviations, indicated by bars attached to symbols, were calculated from the variance of the ratio of the mean numbers of colonies on mutation and survival plates; where bars are absent the standard deviation lies within the area of the symbol. Lines of best fit were obtained from DMS and MNUA data by least-squares linear regression analysis performed on individual mutation plate scores.



a major contribution to the overall mutagenicity of DMS or MMS which produce little *O*⁶-MeGua. Indeed, there is evidence that, in bacteria, MMS is mutagenic by two distinct mechanisms, one of these dependent on the integrity of the *lexA*⁺ (*exrA*⁺)-mediated error-prone repair pathway²². Some insight into the mechanisms of alkylating agent mutagenesis in mammalian cells may be gained by detailed examination of the dose-response curves for mutation induction. The cumulative curves obtained in the present study for *azg*^r mutagenesis could imply the existence of two mutagenic mechanisms, one (presumably *O*⁶-MeGua miscoding) predominating at low doses²³. These curves can be described adequately by equations of the general form $M = aD + bD^2$ ($M = \text{azg}^r$ mutation frequency per 10^5 survivors and $D = \text{dose in } \mu\text{g ml}^{-1}$) and for DMS and MNUA were calculated as $M = 1.30D + 0.13D^2$ and $M = 5.71D + 0.03D^2$ respectively. Interestingly, if we restrict our comparison of mutagenic activity to the linear terms of these equations (that is as D approaches 0) then the numbers of mutants calculated per $\mu\text{mol } O^6\text{-MeGua per mol DNA phosphorus}$ (31 in the case of DMS and 24 for MNUA, extents of methylation estimated from data in Table 1) were very similar for the two agents.

Much remains to be understood about the relationship between specific DNA modifications and the various biological effects of the simple alkylating carcinogens. Nevertheless, the results described here strongly suggest that *O*⁶-MeGua is responsible for the vast majority of gene mutations produced in mammalian cells by carcinogenic methylating agents. Moreover, the correlation between their mutagenicity in mammalian cells

and their documented carcinogenic potency provides further support for the somatic mutation theory of cancer.

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Post-transcriptional control of coordinated ribosomal protein synthesis in *Escherichia coli*

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The synthesis of the approximately 50 different ribosomal proteins (r proteins) is very well coordinated¹ in *Escherichia coli*. As the r-protein genes are arranged in many different operons, placed at separate locations on the *E. coli* chromosome^{2–5}, it is not obvious how this coordination is maintained. The first indication that special controls are involved in the coordination came from the observations that merodiploid strains containing F' factors with some of the ribosomal genes also synthesise the r proteins in balanced amounts^{6,7}. The overall regulation of r-protein synthesis in response to changes in growth conditions is primarily mediated by changes in the rate of transcription of the r-protein genes⁸. To investigate whether the gene dosage control seen in merodiploid strains is also transcriptional in nature or whether other mechanisms are involved, we compared the transcription of r-protein mRNA in haploid and merodiploid strains. It was found that the rate of transcription of the r-protein mRNA from the *str-spc* cluster of genes changes in proportion to the gene dosage and it is concluded that the expression of r-protein genes is adjusted by post-transcriptional control.

About one half of the r-protein genes map in the *str-spc* region at 72 min on the *E. coli* chromosome and the genes are arranged in four separate transcriptional units². All the r-protein genes in the *str-spc* region are present on the F' factor KLF41 and most of the experiments were carried out with a pair of strains, with and without KLF41. Relative rates of synthesis and amounts of r-protein mRNA were measured using different types of hybridisation assays. As probes for different classes of mRNA DNA from the four transducing λ phages was used. λ dfus2 and λ dspc1 carry 27 (all) and 14 r-protein genes from the *str-spc* region, respectively². As control for r-protein mRNA synthesis from genes not duplicated in the merodiploid strain, DNA from a transducing phage, λ E3rp9, carrying r-protein genes from the 88-min region of the *E. coli* chromosome was used. The phage λ E3rp9 was constructed *in vitro* and has the genes for L10, L11 and parts of the genes for L1 and L12 (K. G., in preparation). Nonspecific hybridisation was assayed with λ trk DNA. The phage λ trk is very similar to λ dfus2 and λ dspc1 but it does not carry any ribosomal genes².

For measurements of relative transcriptional activities of the r-protein genes, cultures were labelled in RNA for a period

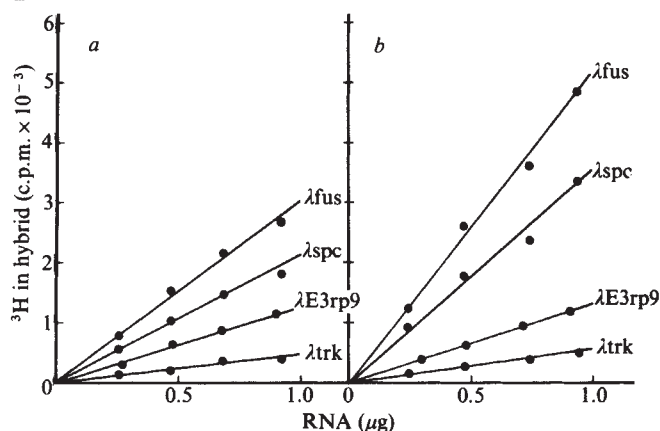


Fig. 1 Hybridisation analysis of pulse-labelled RNA from JC1553 and KLF41/JC1553. JC1553 (ref. 13) (a) and KLF41/JC1553 (ref. 14) (b) were obtained from N. Fiil. The two strains were grown exponentially at 37 °C in AB glucose minimal medium supplemented with a balanced mixture of 20 amino acids, the growth rates were 1.03 generations per h for JC1553 and 0.95 generations per h for KLF41/JC1553. At a density of 2×10^8 cells ml^{-1} the cultures were labelled for 45 s with $[5, 6-^3\text{H}]\text{uridine}$ ($40 \mu\text{Ci ml}^{-1}$ culture, specific activity 40 Ci mmol^{-1}) and the cells were collected on ice with Na-azide (10 mM). The RNA was extracted and hybridised to nitrocellulose filters with 10 μg of DNA from the transducing phages indicated as described previously⁸. Various amounts of RNA (0.25–1.0 μg) were hybridised and the values shown are averages of duplicate samples. The specific activity was 91,000 c.p.m. μg^{-1} for the JC1553 RNA and 88,000 c.p.m. μg^{-1} for the KLF41/JC1553 RNA.

which was short relative to the half life of the unstable RNA fractions. The RNA was hybridised to excess DNA in conditions where the efficiency of hybridisation is better than 90% (Fig. 1). The relative rate of r-protein mRNA transcription from the *str-spc* region is nearly doubled in the F'-containing strain compared with the haploid strain whereas the L1–L12 protein mRNA is transcribed at similar rates in the two strains (Table 1). The gene dosage effect is the same for the complete and half of the *str-spc* region as *spc*-specific mRNA transcription is increased by the same factor as the *fus*-specific mRNA. The precise increase in the gene dosage in the KLF41-containing strain is not known. F' factors are found in one or two copies per chromosome depending on the F' factor and possibly the growth conditions⁹. The relative gene dosage (number of copies of gene *N* per genome equivalent) for the *str-spc* region on the chromosome is 1.15 calculated from the growth rate and the map position¹⁰.

The effect of changing the gene dosage for half of the r proteins in the *str-spc* region was measured by comparing the relative rates of transcription of r-protein mRNA in strains lysogenic for λ dspc1 and λ trk1. λ dspc1 contains two r-protein transcriptional units complete with the promoter regions (14 r-protein genes total)². In the λ dspc1 lysogen the extra set of

Table 1 Relative rate of r-protein mRNA transcription

Strain	% Of total radioactivity			Diploid/haploid		
	L1–L12 mRNA	<i>spc</i> mRNA	<i>fus</i> mRNA	L1–L12 mRNA	<i>spc</i> mRNA	<i>fus</i> mRNA
JC1553	0.79	1.66	2.58			
KLF41/JC1553	0.73	2.92	4.57	0.9	1.8	1.8
NO1294 (λ trk)	0.51	1.36	2.36			
NO1267 (λ dspc1)	0.48	2.10	3.14	0.9	1.5	1.3

The per cent of the radioactivity in pulse-labelled RNA from JC1553 and KLF41/JC1553 that hybridises to four different types of DNA was calculated from the slopes of the curves in Fig. 1. Subtraction of the per cent radioactivity in λ trk DNA hybrid yields per cent radioactivity specific for the r-protein genes on λ E3rp9 (genes for part of L1, L11, L10, and part of L12), λ dspc1 (14 r-protein genes) and on λ dfus2 (27 r-protein genes). NO1294 and NO1267 (from R. Jaskunas) are strains lysogenic for λ trk and λ dspc1. These strains were grown at 30 °C in AB glucose minimal medium; the growth rates were 0.78 generations per h for NO1294 and 0.74 generations per h for NO1267. RNA was labelled and analysed as described in Fig. 1 and results were evaluated as the results for JC1553 and KLF41/JC1553.

r-protein genes is probably located at λatt at 11 min on the *E. coli* chromosome, in which case the gene dosage of the *spc* genes in the $\lambda dspc1$ lysogen is 1.7 relative to that in the $\lambda dtrk$ lysogen. The increase in the relative rate of transcription from the duplicated genes is 1.5 (Table 1), which is seen directly from the *spc* mRNA results. The *fus* mRNA results are compatible with a 1.5-fold increase in the rate of transcription from the genes in the *str-spc* region that are duplicated in the $\lambda dspc1$ lysogen and identical rates of transcription from the genes that are not duplicated. A similar gene dosage effect on the rate of transcription of r-protein mRNA has been reported for a related $\lambda dspc1$ lysogen¹¹.

The JC1553 pair of strains differ from the pair of lysogens not only with respect to the mode of duplication of the r-protein genes. JC1553 is *recA* which is reflected in the relative low growth rate (see Fig. 1 legend), whereas the lysogens are *recA*⁺. However, analysis of both sets of strains leads to the conclusion that the rate of transcription of the genes in the *str-spc* region is nearly proportional to the gene dosage. As the rate of synthesis of the r proteins in the merodiploid cells is well coordinated and identical to the rate of synthesis in the haploid cells^{6,7,11}, it follows that the yield of peptides per round of transcription is reduced specifically for the mRNA from the duplicated genes.

We then asked if this decrease in translation efficiency is reflected in the stability of these mRNAs. When the relative rate of transcription of a class of mRNA is known the average physical half life can be deduced from estimates of the amounts of mRNA. The relative amounts of r-protein mRNA were therefore determined in JC1553 and KLF41/JC1553. In one experiment the two strains were labelled uniformly in RNA and the per cent radioactivity in different classes of r-protein mRNA was measured by hybridisation to excess DNA. Table 2 shows that the merodiploid strain contains elevated levels of mRNA from the duplicated regions but the genes dosage effect is not as pronounced as for the rates of transcription of these mRNA species (Table 1). In another experiment pulse-labelled RNA was hybridised to limiting amounts of DNA. When DNA-RNA hybridisation approaches saturation a linear curve is obtained in a reciprocal plot (Fig. 2) and it has been shown that the amount of DNA-specific RNA is inversely proportional to the slope of the line¹². Also this experiment shows that a duplication of r-protein genes is accompanied by a significant increase in the amount of the corresponding r-protein mRNA. A smaller increase (20%) in the amount of *spc* mRNA was seen in a strain

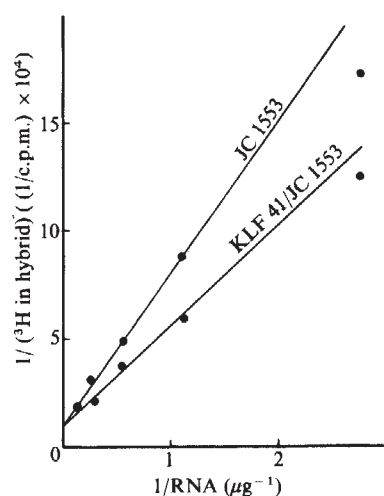


Fig. 2 Relative amounts of r-protein mRNA determined by hybridisation to limiting amounts of DNA. Different amounts of pulse-labelled RNA (0.4–7.0 μg) from JC1553 and KLF41/JC1553 (the same samples as used in Fig. 1) were hybridised to nitrocellulose filters with 0.5 μg $\lambda dtrk$ DNA and 0.5 μg $\lambda dfus$ DNA. Values on the ordinate represent c.p.m. hybridised to $\lambda dfus2$ DNA corrected for c.p.m. hybridised to $\lambda dtrk$ DNA. The reciprocal of the slope for KLF41/JC1553 is 1.6 $\times$ the reciprocal of the slope for JC1553.

Table 2 Amounts of r-protein mRNA

Strain	JC1553	KLF41/JC1553
Total c.p.m.	154,000	158,000
c.p.m. In hybrid:		
$\lambda dtrk$ DNA	510	575
$\lambda E3rp9$ DNA	637	685
$\lambda dspc1$ DNA	858	1,098
$\lambda dfus2$ DNA	1,259	1,591
% In mRNA:		
L1–L12	0.08	0.07
<i>spc</i>	0.23	0.33
<i>fus</i>	0.49	0.64
Diploid/haploid:		
L1–L12 mRNA	0.9	
<i>spc</i> mRNA	1.44	
<i>fus</i> mRNA	1.31	
<i>fus</i> mRNA	1.6 (from Fig. 2)	

JC1553 and KLF41/JC1553 were grown exponentially at 37 °C in MOPS minimal medium with low phosphate (0.2 mM). The growth rate was 0.76 generations per h for JC1553 and 0.73 for KLF41/JC1553. At a density of about 6×10^6 cells ml^{-1} ^{32}P was added to the cultures (10 μC ml^{-1}) and 5 generations later (at 2×10^8 cells ml^{-1}) the cells were collected. RNA was extracted and duplicate portions of 0.5 μg and 1.0 μg were hybridised to nitrocellulose filters with 10 μg DNA. The c.p.m. in hybrid listed represent average values.

lysogenic for a $\lambda dspc$ phage¹¹, which probably reflects that the increase in r-protein gene dosage is smaller in the lysogen than in the KLF41-containing strain.

As the rate of transcription of the *str-spc* mRNA is increased more than the amount of the *str-spc* mRNA in the merodiploid strain, it follows that the average physical half life of the mRNA from the duplicated r-protein genes is somewhat shorter than in the haploid strain. However, the merodiploid strain does contain an excess of mRNA sequences from the duplicated r-protein genes.

In conclusion we have found that the rate of transcription from the r-protein genes in the *str-spc* region increases in proportion to the gene dosage when extra copies of these genes are supplied by an F' factor or a transducing λ phage. As the rate of synthesis of the r proteins is unaffected by such changes in gene dosage^{6,7} it shows that post-transcriptional controls adjust the rate of r-protein synthesis.

On the basis of similar observations it was recently proposed that the post-transcriptional control acts by inactivation and degradation of the excess of r-protein mRNA¹¹. The results available so far are, however, equally compatible with mechanisms that involve blockage of translation of intrinsically active r-protein mRNAs. The increased lability of overproduced mRNAs could then be the consequence rather than the cause of their lower degree of participation in protein synthesis. The precise coordination of r-protein synthesis in normal cells, coupled with the recent observations that the r-protein gene clusters are spread over most of the chromosome^{2–5}, suggests that post-transcriptional control may be evolved in normal cells in order to compensate for the successive replication of the different r-protein genes during the cell cycle.

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MATTERS ARISING

Hybridisation of scDNA does not lead to overestimates of mRNA complexity

THE contention of Kiper¹, that complexity estimates of mRNA populations measured by hybridisation with single copy DNA (scDNA) are routinely overestimates, is not supported by previously published data and additional data we provide here. Kiper argues that selective loss of single copy sequences (cryptic unique sequences) occurs when scDNA is isolated from randomly sheared DNA because they are adjacent to repetitive sequences. He contends that the loss is selective for sequences which do not code for messenger RNA, and hence scDNA is enriched in sequences which code for mRNA. Thus complexity measurements on mRNA based on scDNA hybridisation result in overestimates.

From data on the sea urchin genome² it can be calculated that ~19% of the total genomic DNA might be present as 'cryptic' scDNA when 450-nucleotide fragments are used in the preparation of scDNA. Assuming all of the cryptic scDNA to be non-code sequence, the resulting scDNA would be enriched for code sequences such that saturation hybridisation data might yield a complexity estimate that is too high by a factor of ~1.3, which is less than suggested by Kiper. A more important consideration is the likelihood that code sequences are extensively represented in cryptic scDNA. For example 80–100% of the mass of the mRNA in sea urchin embryos is complementary to scDNA sequences which are adjacent to interspersed repetitive sequences³. If this is also true for complexity, and if the sequence organisation of sea urchin DNA is typical of eukaryotic genomes, then cryptic scDNA would contain code sequences and may even be somewhat enriched for these sequences. Whatever the case, a portion of the cryptic scDNA is expected to be code sequences. We have also found, using S₁ nuclease, that only about 10% of mouse DNA considered to be repetitive by hydroxyapatite (HAP) analysis of renaturation kinetics may be cryptic scDNA. Thus the extent of code sequence enrichment of scDNA suggested by Kiper seems unlikely.

Kiper's second point, that assay of DNA/RNA hybrids by HAP chromatography leads to overestimates due to the non-base-paired intervening sequence being present in DNA/RNA hybrids, is an important consideration in light of recent studies which suggest that several known structural genes are discontinuous in the genome^{4–6}, and perhaps, as well, a large

number of unidentified mRNA coding sequences⁷. However, values obtained by HAP and S₁ nuclease assays of hybridised scDNA are similar (Table 1). It is evident from this comparison that a negligible portion of the hybridised scDNA is not base paired, as has been previously reported⁸. Despite these results we concur that S₁ nuclease method of assay is preferable to the use of HAP for reasons presented elsewhere⁹.

From the aspects mentioned above Kiper suggests that the discrepancy between the scDNA and the cDNA methods of estimating the complexity of mRNA populations is largely due to gross overestimates generated by the scDNA method. From our results we find the discrepancy to be due more to the inaccuracy of the C₀t^{1/2} determinations for the most complex class of mRNA which is particularly imprecise if the cDNA is only partially hybridisable (that is, ~75% or less). We prepared cDNA complementary to mouse brain poly(A)⁺mRNA which was hybridisable to 91–92% S₁ resistance. From hybridisation kinetics the complexity of the poly(A)⁺mRNA was estimated to be 1.1 × 10⁵ kilobases¹⁰, a value which is similar to that obtained by the scDNA hybridisation method (Table 1). We conclude that the scDNA hybridisation method does not lead to gross overestimates of the complexity of RNA populations for reasons offered by Kiper.

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HYBRIDISATION of labelled single copy DNA with an excess of RNA is an important procedure for estimating the number of genes expressed in cells¹. Recently, Kiper² asserted that gene number values in plants obtained by using this method are routinely significant overestimates. To support his premise, Kiper cited our measurements of the

number of genes expressed in the tobacco leaf³. We contend that our original interpretation of the tobacco data³ is theoretically sound and that the re-interpretation by Kiper² is based on specious arguments.

Kiper's first argument maintained that a large fraction of interspersed single copy DNA (scDNA) in plant genomes consisted of ≤300-base pair, unexpressed sequences. During the kinetic isolation of scDNA, these short, interspersed, unique sequences would be selectively lost due to their close linkage with repetitive sequence elements. The effective result (as Kiper maintains), therefore, would be an enrichment for expressed sequences in the single copy preparations used for DNA/RNA hybridisation experiments. Most plant genomes investigated to date, however, do not possess very short interspersed single copy sequences⁴. For example, the vast majority of interspersed single copy sequences in tobacco⁵, soybean^{6–8}, cotton⁹, corn (S. Hake and V. Walbot, unpublished), wheat¹⁰, and mungbean¹¹ exceed 1,000 base pairs. Only in pea¹² and perhaps rye¹³ does there seem to be a fraction of short scDNA. Direct electron microscopic observations have shown that the minimum number average lengths of interspersed single copy sequences in soybean⁷ and cotton⁹ are 1,300 and 2,900 base pairs respectively. Few short (<500 base pairs) single copy sequences were visualised (<5%). As the fragment length (200–300 bases) of scDNA used in gene expression studies is much less than that of the interspersed unique DNA, it is unlikely that a separate complexity class of unexpressed, 'cryptic', single copy sequences will be removed during scDNA isolation. The scDNA mass that is lost (due to short period interspersed effects) will simply be that which is at the termini of moderately long scDNA regions. Such regions have been demonstrated to code for mRNA^{14–16}. We argue, therefore, that short plant scDNA preparations of the type used in RNA-excess/scDNA hybridisation experiments will not be enriched for expressed sequences or any other sequences as Kiper asserts.

Kiper's second argument was based on recent observations which have demonstrated that several animal genes are interrupted by non-coding sequences (intervening sequences)¹⁷. He contends, correctly, that if intervening sequences are present in plant genomes, they could potentially lead to an overestimate of gene number when DNA/RNA hybrids are assayed by binding to hydroxyapatite. However, as shown in Table 2 this is not the case, as similar gene number values were obtained with the scDNA method

Table 1 Complexity of RNA from mouse brain assayed by HAP or S₁ nuclease

RNA	Method of assay*	scDNA as hybrid (%)	Complexity‡ (kilobases)
Poly(A) ⁺ mRNA, brain	S ₁ nuclease	3.9	1.25 × 10 ⁵
Poly(A) ⁺ mRNA, brain	HAP	4.1	1.33 × 10 ⁵
Poly(A) ⁺ mRNA, brain	HAP	3.6†	1.17 × 10 ⁵
Nuclear, brain	S ₁ nuclease	18	5.85 × 10 ⁵
Nuclear, brain	HAP	19	6.17 × 10 ⁵
Nuclear, brain	HAP	19.9†	6.46 × 10 ⁵

* Assays were as previously described^{8,9} with slight modifications.

† Previously reported value⁸.

‡ Complexity estimate calculated on the basis that the single copy fraction of the mouse haploid genome is 3.25 × 10⁹ nucleotides.

using both S₁ nuclease (which would eliminate non-coding sequence effects) and HAP assays. These data demonstrate that the presence or absence of intervening sequences has an insignificant effect on gene number values obtained by using HAP analysis with short single copy tracers.

In support of his arguments Kiper compared values obtained from mRNA/cDNA hybridisation studies¹⁸ with those derived from RNA/single copy analysis¹. In the case of our data³ Kiper noted 'strikingly different' estimates of the number of genes expressed in the tobacco leaf from the two procedures (12,000 against 27,000). In our original report we discussed the inaccuracies inherent in gene number values derived from cDNA hybridisation. We stated explicitly that the data were consistent with a range of 6,000 to 24,000 diverse gene transcripts (see Fig. 3 and Table 1 of Ref. 3) and were thus consistent with the value obtained from the single copy procedure (see Fig. 4 and Table 2 of Ref. 3). As we and others¹⁹⁻²² have discussed, computer solutions of cDNA hybridisation results must be interpreted with circumspection. In the absence of other information (for example, hybridisation analysis of isolated rare message cDNA), the gene number values computed cannot be considered unique and are accurate to only within a factor of 2.

Even if there are only 12,000 diverse gene transcripts represented in the tobacco leaf poly(A)⁺mRNA population, this value would still be compatible with 27,000 gene transcripts in total mRNA. Approximately 50-70% of leaf mRNA is non-adenylated^{3,23}. Several laboratories²⁴⁻²⁶ have demonstrated that poly(A)⁺ and poly(A)⁻ mRNAs may contain distinct subsets of gene transcripts. For example, there are about 120,000 diverse gene transcripts present in the poly(A)⁺ mRNA of mouse brain cells compared with 220,000 in total mRNA^{24,27}. Thus, a comparison of cDNA hybridisation data with scDNA hybridisation data cannot be used to support Kiper's contention that the latter method overestimates gene number.

Finally, we wish to point out that gene number values, as estimated by any pro-

cedure, cannot be considered absolute. They are limited by many factors, including the presence of undetectable rare mRNAs (<0.1 molecule per cell), the use of abundant mRNA sizes to calculate gene numbers²⁸, salt correction factors used in kinetic analysis (W.E.H. unpublished) and the precision in measuring very small proportions of hybridised DNA. At the present, however, single copy measurements produce the most precise and reliable estimates of gene numbers, even in higher plants.

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KIPER REPLIES—The first point stressed in my recent paper¹ was that in eukaryotic single copy DNA prepared for mRNA complexity measurements short unique sequences are absent and ends of longer unique sequences are under-represented while long unique DNA sequences will be over-represented. This is caused by sequence interspersions of different kinetic classes and random shearing. Thus taking the total nuclear unique DNA content as a basis for complexity calculations, gene number overestimates result if mRNAs are preferentially encoded for by longer unique sequences.

Concerning the sea urchin mRNA complexity measurements, the degree of over-representation could indeed be 1.5 as could the gene number overestimate: so, at a length of 450-nucleotide reassociation kinetics displayed a 50% unique DNA content². Galau *et al.*, however, took a 75% content as the basis for their estimates³, a value derived from the fraction of S₁-digestible C₀t 20 reassociated DNA².

The point questioned by Goldberg and Timberlake is whether genomes (especially of the plant kingdom) will contain gross amounts of short cryptic unique DNA. This, now, is certainly proved for pea DNA. Murray *et al.* demonstrated that the major class of single-copy sequences has a modal length of about 300 nucleotides (ref. 4) and that due to the intimate interspersions with repetitive DNA no true single copy tracer can be prepared⁵. Goldberg and Timberlake's contention is that in tobacco (and other plants) very few unique sequences are shorter than 500 base pairs and the average length of interspersed scDNA sequences is at least 1,400 nucleotides. Hence the scDNA preparation is a fair representation of the total scDNA complexity, cryptic DNA thus becoming negligible. Their data, however, seem to conflict with this view: so, in HAP renaturation kinetics analysis of a fragment length of 300 nucleotides 22-23% of the whole genome was included in the unique DNA fraction. This increased to 43% using S₁ nuclease (47% after some minor correction)⁶. Their mRNA complexity measurements were based on the complexity of a 42.5% scDNA fraction⁷. Now think of a population of interspersed unique DNA sequences averaging 1,400 nucleotides; then during reassociation analysis of sheared 300-nucleotide-long whole genomic DNA, 81% of this would be scored as unique DNA using HAP analysis (for formulae see ref. 1): that is a

Table 2 Estimates of gene number derived from the single copy procedure using hydroxyapatite and S_1 nuclease assays

Organism	Assay		Ref.
	HAP	S_1 nuclease	
Tobacco	27,000	24,000	3*
<i>Achlya</i>	2,000	3,200	29, 30
Mouse brain	100,000	120,000	24, 27

*J.C. Kamalay and R.B.G., unpublished.

23% increase would be expected, revealing the whole unique DNA content using S_1 nuclease. The 87% increase found in tobacco (taking the corrected value of 47% instead of 43% this amounts to a more than 100% increase) seems rational only at an average unique DNA fragment length of less than 600 nucleotides. As many sequences seem to be longer than 1,000 nucleotides this favours a broad class of rather short unique sequences behaving cryptically. Only if all these unique sequences—whether short, medium or long—code for mRNA to the same extent would it be valid to base gene number estimates of tobacco and other organisms on the total nuclear unique DNA complexity. However, this is unlikely considering what is now known about mRNA length and organisation of individual genes.

Hahn *et al.* argue that coding sequences are extensively represented in a repetitive sequence neighbouring unique DNA, which is, as I pointed out, under-represented in scDNA preparations; nevertheless it would still be valid to refer to the total unique DNA complexity. In support of this they cite the sea urchin experiment of Davidson *et al.*⁸. However, the mRNA mass-complexity equivalent postulated for this experiment is not backed by any results. In that experiment 77% of the mass of all mRNA seemed to be adjacent to interspersed repetitive sequences; but, as only a total of $8 \pm 4\%$ of all mRNA mass represented almost the whole complexity³, the results can equally be interpreted as meaning that only a few (abundant) mRNAs were adjacent to repetitive DNA. Furthermore, the HAP assay was used, and in view of recent insights into gene interruption this technique could have measured minor coding portions of genes adjacent to repetitive elements. The main argument, however, is that the last fractionation in purification of the repeat contiguous DNA excluded all unique DNA behaving cryptically in reassociation kinetics from the pool which was allowed to hybridise with the mRNA population. Therefore, this experiment is irrelevant to the question.

The finding of Hahn *et al.* that in mouse only 10% of HAP-assayed repetitive DNA is digested by S_1 nuclease is in contrast with other findings, for example, in the sea urchin² and tobacco⁶ where 50% and 43%, respectively were digestible. Thus the mouse experiments do not

seem to be representative and favour minimal interspersion between different kinetic classes in the mouse genome.

Concerning intervening sequences—the second point of my paper—their general occurrence will have to be deduced by further research. A close look at the organisation of individual genes, as carried out for the globin genes⁹ or the ovalbumin gene¹⁰ and the ovomucoid gene¹¹ and lysozyme¹², has revealed the existence of rather short gene domains separated by intervening sequences of about equal length or longer. If the intervening sequences are unique in the genome too, as shown for the globin¹³ and the ovalbumin gene¹⁴, that is if genes are located in long stretches of unique DNA, a large fraction of the introns will be scored as coding complexity in the scDNA saturation experiments described. Only if the length of the scDNA preparation is much smaller than the exons would interference by intervening sequences be unimportant. However, most of the coding domains known today are even shorter than the scDNA preparations routinely used in complexity measurements^{9–12}. The data compiled by Goldberg, Timberlake, Hahn and coworkers in their critiques seem to contradict the argument. Apparently, however, to solve the problem it will be necessary to check HAP-isolated hybrids for their content of non-coding single-stranded DNA loops.

Previous interspersal analysis of many genomes has demonstrated that sea urchin DNA organisation is not typical of eukaryotic genomes as postulated by Hahn *et al.*; nor is any other sequence organisation pattern. Eukaryotic genomes differ largely in their content of repetitive DNA and their interspersal patterns. Long period interspersal of unique DNA was found to be predominant in birds¹⁵, fungi¹⁶ and insects¹⁷. Higher plants show varying interspersal patterns with unique DNA lengths of 300 nucleotides being predominant in pea⁵ while *Xenopus*-like patterns are predominant in tobacco⁶. Short unique sequences can be separated by short repetitive sequences (300–400-nucleotide fragments in pea⁶), repetitive sequences 1,250 base pairs long (as in cotton¹⁸) or by repetitive sequences thousands of base pairs long, as in the genome of pearl millet¹⁹. Higher plants with low haploid genome size contain half of their scDNA in stretches a few thousand base pairs long^{19,20}. Most other plant and animal genomes also contain long unique DNA sequences. So, the question remains whether coding sequences are typically located within long stretches of unique DNA.

However, genome and gene organisation complicate interpretation of DNA saturation experiments. Furthermore, many considerations lead to the conclusion that scDNA saturation experiments will routinely overestimate

numbers of active genes: over-representation of coding sequences in the unique DNA preparation, split genes, few-copy DNA taken for scDNA, gene duplication as in the human globin genes, cross hybridisation of certain gene domains²¹, and scoring of unique gene flanking sequences as coding. So, many detailed answers have to be given before the results can be interpreted with confidence. This will be a more severe problem with genomes containing large amounts of repetitive DNA and extensive interspersal with unique DNA, as is typical in higher plants.

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Aminostratigraphy of Pleistocene and Holocene deposits

RECENT work on amino acid ratios for sites in the UK is a major step forward in the research of the Pleistocene and early Holocene periods^{1,2}. Archaeological work, coupled with environmental studies, can be valuable in supporting amino acid research or in pinpointing possible discrepancies and problems. Miller *et al.*¹ have compared the Purfleet and Swanscombe Pleistocene sites, pointing out that the mean D-alloisoleucine: L-isoleucine ratio for Purfleet is 0.35 while for Swanscombe the ratio is 0.30. At Purfleet, as at Swanscombe, there are three main gravels separated by sands and silts, and the artefacts from Purfleet are comparable with those of the Middle Gravels from Swanscombe³. The shell samples used from

Swanscombe came from the Middle Gravels but those from Purfleet came from a shell band which is ~1 m or more below the main or Middle Gravel at that site, immediately above a coarse sandy orange-brown gravel, referred to as Gravel 3. Very few artefacts came from this Lower Gravel, but some of Clactonian aspects were present. It can, therefore, be suggested that the whole series of deposits at Purfleet are comparable to those at Swanscombe. As the amino acid ratios from Purfleet refer to deposits which are stratigraphically somewhat older than the Middle Gravel at Swanscombe, the discrepancies in the ratios can possibly be explained on this basis as dating slightly different phases of the same interglacial.

Andrews, Bowen and Kidson² pointed out that the Portland Raised Beach and Burtle Beds have produced ratios which do not agree exactly with those of Middle Hope and Minchin Hole, all frequently assigned to the last interglacial. There may be several explanations for these discrepancies. Attention has often been drawn to the fact that there are pronounced altimetric differences between the Beach deposits in various parts of the Bill area of Portland, and it has recently been pointed out that there may be at least two quite distinct raised beaches on the Isle⁴. Here again, different phases of the same interglacial may be involved, indicating minor advances and regressions of the sea.

On the other hand, other environmental factors could possibly be involved, as suggested by the ratios of the Holocene sample from Portland. This sample came from the Mesolithic habitation site of Culver Well, with a very large shell-midden with uncalibrated radiocarbon dates of 5200±135 BC (BM-473) and 5151±97 BC (BM-960); a weighted average of three samples of burnt limestone from a hearth has given a thermoluminescence date of 5400 BC±640 (OxTL 501 b, m)⁵. The stratigraphical evidence and archaeological finds from the midden clearly indicate a dating within the Mesolithic period, falling within the Atlantic climatic zone. The other Holocene sample came from Skara Brae in the Orkneys, a site known from the archaeological evidence to belong to the late Neolithic period associated with pottery of the Rinyo type, that is this site is at least 3,000 yr younger than Portland (or less than half the age of the Mesolithic site). Yet both sites have produced D-allo: L-iso (combined) ratios of 0.04, indicating that results from one or the other, or perhaps even both, the Holocene control samples are unreliable. This too could possibly be explained by different environmental or economic circumstances relating to the sites, during the relevant prehistoric periods and after, affecting the epimerisation reaction of the samples. Or it may be that the sensitivities of the techniques are not yet so advanced that

differences of only a few thousand years can be registered.

These discrepancies do not, however, minimise the great value of the work being done on amino acid reactions as potential chronological indices.

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MILLER ET AL REPLY—Our *Corbicula* from Swanscombe are not from, but merely correlated with, the Middle Gravels there. If the correlation is correct, then the absence of *Corbicula* in the Swanscombe Lower Loam and Lower Gravel, at an elevation intermediate between the Middle Gravels and Grays (with similar ratios), suggests that the Lower Loam and Lower Gravel may be from an older interglacial. The *Corbicula* at Purfleet could be from early in the Middle Gravels interglacial, or from another interglacial, intermediate in age between the Middle Gravels and Lower Loam–Lower Gravel ones.

At current temperatures in Britain, amino acid epimerisation occurs slowly; hence, as Palmer infers, we cannot detect as yet differences of a few thousands of years. This does not mean that such discrimination is not possible. Instrumentation is improving rapidly, and there are several different reactions that may give the resolution required.

Ours^{1,2} are preliminary analyses: our papers have demonstrated that amino stratigraphy will provide a useful time-frame for UK Pleistocene studies. To that end, we would like to say that we are very willing to cooperate with UK researchers on problems where this technique may be applicable.

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Asymmetry in relatedness: who is related to whom?

FLESNESS¹ has brought out the interesting fact that relatedness between diploids can be asymmetric, as it is between the sexes of male-haploids². His note, however, contains a conceptual difficulty.

This concerns the directionality of relatedness in his otherwise valuable definition. Because relatedness calculations are usually made in order to determine the (theoretical) optimum course of action of a potential altruist *vis-à-vis* a particular recipient, we suggest that the quantity implied in the phrasing 'relatedness of recipient to donor' should be the genetic similarity of the recipient to the donor, but under Flesness's definition it is the genetic similarity of the donor to the recipient. If weighing up (in terms of relationship) the benefits of being altruistic towards someone, you should care about the proportion of your genes that is present in him, and not the portion of his genes present in you. We therefore re-define Flesness's coefficient:

$$R_{B(A)} = \text{relatedness of B to A}$$

$$= \frac{(\text{mean identity by descent of A's genes with B's gametes})}{(\text{mean identity by descent of A's genes with A's gametes})}$$

This directionality is also the same as that adopted by Hamilton³ in discussing essentially the same parameter. As a consequence, the values given in Flesness's table should be reversed.

$R_{B(A)}$ (as defined above) has the same meaning as the 'pedigree coefficient of relatedness', $G_{B(A)}$ (ref. 2, and R. Frankham, in preparation), and is also similar to the 'regression coefficient of relatedness', $b_{B(A)}$ (ref. 3) (with the subscript order reversed here to conform with standard regression notation). We suggest that $G_{B(A)}$ be retained for use as the central measurement of relatedness in pedigrees (as $R_{B(A)}$ is more easily confused with Wright's⁴ 'coefficient of relationship', r), and that Hamilton's coefficient be retained for estimates of relatedness derived by regression analysis of populations (P.P. and R.H.C., in preparation).

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BOOK REVIEWS

Enigmatic variations

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THIS well produced paperback addresses the question as to what influence solar variations have on weather and climate. This is a highly controversial and (as I know from personal experience) an emotional subject.

So let me, at the outset, lay my own established position on the line. Quoting from my published review "... it seems that despite a massive literature on the subject, there is at present little or no convincing evidence of statistically significant or practically useful correlations between sunspot cycles and the weather or climate." (*Rev. Geophys. Space Phys.*, 16, 400; 1978). At the time I wrote that (late 1977), swayed particularly by the critical examination by C.O. Hines and I. Halevy (*J. Atmos. Sci.*, 34, 382; 1977) of the work of J.M. Wilcox *et al.* (*J. Atmos. Sci.*, 33, 1113; 1976), I conceded that there were "... apparently significant short-term correlations between meteorological indices and the passage of solar magnetic sector boundaries...". Now that I have read R.G. Williams and E.J. Gerety (*Nature*, 275, 200; 1978) and R. Shapiro (*J. Atmos. Sci.*, 36, 1105; 1979) on that particular subject I am not so convinced by the short-period effects either.

Contrast these rather negative comments with the more positive upbeat approach and conclusions of Herman and Goldberg. In the preface (page v) we read: "Even though the reported results have sometimes been confused, disjointed, and contradictory, there has emerged a growing belief that there are connections between changes on the Sun and changes in the lower atmosphere". In the penultimate chapter (page 274) entitled "Recapitulation of Sun-Weather Relationships" we read that "In summary, the correlations between solar activity and meteorological responses are highly suggestive and require further investigation. To take an agnostic point of view, these relationships have not been proven or disproven and therefore require further attention."

What accounts for this apparently profound difference in our assessments of essentially the same evidence? I wish the philosophers of science would address themselves to this particular controversy because I suspect that in the literature on the solar weather/climate relationships they will find their best possible case study of objectivity versus subjectivity, and of the conscious or unconscious influence of

Sun, Weather, and Climate. By J.R. Herman and R.A. Goldberg. Pp.360. (National Aeronautics and Space Administration: Washington, DC, 1979.)

vested interests and disciplinary prejudices. It is for instance almost axiomatic in this controversy that meteorologists and climatologists will applaud my 'critical' approach, while ionospheric and solar physicists will prefer Herman and Goldberg's more optimistic or 'agnostic' approach.

The cynics will put this down to blatant self-interest, each group seeking to justify its own research interests and even secure funding for its more expensive projects. But I believe it runs deeper and is more subtle than that. Whatever the motivation, the disbelievers and skeptics demand a degree of statistical rigour and level of proof which the believers feel to be unnecessary or destructive of a potentially important physical and practical insight into nature. The protagonists can point to the well established proven influence of solar variations on the ionosphere and mesosphere and suggest that such influence must extend down into the meteorologically active regions. The presumption is that such effects exist in the lower atmosphere and the problem lies in establishing the magnitude and precise nature and mechanisms of such effects.

What we are seeing in this controversy is thus a reversal of the onus of proof or of the null hypothesis from that which is usual in science. The protagonists wish to use as their null hypothesis the proposition that solar effects do exist, and are thus satisfied (at least for now) with a situation in which this has not been disproven beyond reasonable doubt. The opposite null hypothesis, that no effect exists until it is proven to exist beyond a reasonable doubt is the traditional hypothesis which safeguards the purity of established scientific theory. In the present state of the solar weather/climate controversy these two positions are poles apart in terms of the level of proof or rigour applied to the available data.

To take a couple of specific (if perhaps extreme) examples from Herman and Goldberg's book, let us look at their treatment of two of the more spectacular and well known claims of a significant effect of solar cycles on climate.

Herman and Goldberg report concerning thunderstorm frequency (page 142) "The strongest correlation [with sunspot numbers] (about + 0.9) is found in Siberia and has been consistently reported as such for many years (Septer, 1926; Shaw, 1928; Brooks, 1934). Likhter reported the same value again in 1968 (Markson, 1971)." [*Pure Appl. Geophys.*, 84, 161; 1971]. They then go on to discuss these results, stating that the "original work on Siberian thunderstorms was apparently done by Septer" and noting that Brooks used Septer's data, but not making clear that Shaw also used Septer's data and that Likhter *et al.* did not find such a strong relationship using more recent data. Markson's reference to Likhter *et al.* (1968) is to an unpublished conference paper, but a paper with the same authors and title is Likhter, Ia.I., Kolokolov, V.P. and Kleimenova, E.P.; *Trudy, Glavnaia Geofiz. Abs.* No.242, 104; 1969. They used IGY-IQSY data and found a more "complex relationship" than that reported by Brooks. Instead of four independent and consistent results we thus have only one, which is not so impressive. Herman and Goldberg compound this inflated impression of Septer's results by failing to note that in fact later surveys by H.J. Fischer and R. Mühleisen (*Meteorol. Rundsch.*, 25, 6; 1972), and Z.P. Kleimenova (*Meteorol. Gidrol.*, No.8, 64; 1967) using more recent data fail to find significant positive correlations between thunderstorm frequency in Siberia and sunspot numbers. Indeed Kleimenova reveals that she could not find records from as many stations as Septer claimed to have used. Kleimenova then attempted to verify Septer's result with all the available data for the same period and concluded that "the results obtained by Septer are not in accord with fact...". How unfortunate that one of the most spectacular and oft repeated claims in the literature rests on data which can no longer be found! What weight can we give to this claim?

The second example is the authors' treatment of the claims by Xanthakis for large and significant positive or negative correlations between zonal indices of precipitation and sunspot activity. Herman and Goldberg here faithfully report the reversals of sign in correlation coefficients between zones and between epochs in the same zones and also note the inconsistencies of J. Xanthakis' results (in *Solar*

Activity and Related Interplanetary and Terrestrial Phenomena; Springer: New York; 1973) with those of E.S. Gerety *et al.* (*J. Atmos. Sci.*, **34**, 673; 1977) and M. Dehsara and K. Cheak (*Arch. Met. Geoph. Biokl.*, Ser. B, **18**, 253; 1970). But they seek to explain these, not by a critical examination of the conclusions of, methodology, and data sets used by Xanthakis, but by appealing to a more complex hypothesis allowing more degrees of freedom. Thus on page 130 they say "If the pattern of the global distribution of excess rainfall . . . undergoes secular changes due to long-term variations in extraterrestrial or even terrestrial influences, averages over wide geographic expanses could very well exhibit correlation reversals with sunspot number . . . Analysis along (these) lines might reconcile some of the discrepancies . . .".

This is a common argument used by the protagonists of solar effects but in the absence of specific theories as to how things ought to change in time or space it simply allows infinite room to manoeuvre, 'explaining' all inconsistencies and proving nothing. It is to the authors' credit that they make this argument fairly explicit in a section headed "Correlation Reversals and Failures", but it is nevertheless a dangerous argument since it removes the incentive towards a truly critical examination of the claims in the literature. As if to prove my point, subsequent admissions by J. Xanthakis (*Nature*, **280**, 254; 1979) that he in fact selected his data *a posteriori* have now clearly invalidated the significance of his claimed results.

Similar criticisms can be made of the authors' rather uncritical acceptance of many other contradictory claims in the literature, for example in their discussions of much of King's work, including King's treatment of rainfall at Fortaleza (pages 119-120) and of Bowen's claims for Australian rainfall (pages 88 *et seq.*).

So far my comments have been rather negative. However I want to pay tribute to Herman and Goldberg for several important things. One is the comprehensive (if not complete) nature of their survey and its clear presentation. In particular the excellent indexing greatly enhances the value of the book. Their highlighting of some of the important inconsistencies and breakdowns in the reported correlations is another big plus.

Perhaps the greatest virtue of the book is its emphasis on the need to develop and test physical theories without which the study of solar-weather/climate relationships will never emerge from its present morass. This emphasis will be found in the authors' very first sentence (Preface, page v) and is substantiated in a chapter devoted to possible "Physical Processes and Mechanisms" (chapter 6) and in the final chapter "Guidelines for Experiments" (chapter 8). Inevitably, coming from people deeply involved in research in relevant areas, this emphasis seems to be rather self-serving.

Nevertheless it is a necessary and correct general emphasis, whatever we may think of their particular suggestions and their too ready acceptance of apparently relevant but dubious claims in the literature.

In summary then, I find Herman and Goldberg's book to be comprehensive (although not perfectly so) but regrettably uncritical in its approach to the literature. It has a desirable emphasis on the need for physical theories and explanations, and on attempting to isolate critical experiments. It also draws together information concerning a wide range of possibly relevant phenomena and processes. Although the case for rejecting the null hypothesis that there are no significant effects has not been proven by this book,

there nevertheless remains a case for supporting further research directed with physical insight at the hypothesis that there just might be significant effects. We might at least usefully attempt to see how far down such effects penetrate into the atmosphere and to set upper limits on their magnitude in the troposphere. Read with healthy skepticism and critical discernment, Herman and Goldberg's book makes a useful contribution to such an effort. □

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Kinetoplastid biology

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Biology of the Kinetoplastida. Vol. 2. Edited by W.H.R. Lumsden and D.A. Evans. Pp. 738. (Academic: London, New York and San Francisco, 1979.) £45; \$94.

VOLUME 1 of this two-part work is an invaluable reference on trypanosomes and leishmanias, serving as an 'in-filler' of gaps in other standard works as well as presenting new developments in better-known fields. The present volume seems certain to follow suit. Its net is cast wider than its predecessor's, but the emphasis of its fifteen chapters is on parasites of man and domestic animals.

J.J. Shaw and R. Lainson review the ever-changing scene of leishmanial taxonomy and epidemiology and introduce three subgeneric "sections". R. Killick-Kendrick fully discusses *Leishmania* in its vector; and V.P. Sergiev describes the epidemiology of leishmaniasis, now mainly zoonotic, in USSR. M.A. Miles discusses biochemical and immunological heterogeneity of *Trypanosoma cruzi* and the problems arising therefrom; and its pathogenesis and biochemistry are

reviewed respectively by W.L. Tafuri and by W.E. Gutteridge and G.W. Rogerson. W.E. Ormerod considers old and new concepts of the life cycle of *T. brucei* in mammals and heretically suggests that it is not the "stumpy" form which infects tsetse, and that a special "tissue phase" exists. W.J. Herbert and D. Parratt stimulatingly discuss virulence ("the capacity . . . to damage . . . its host") and heterophile antibodies in (almost exclusively African) trypanosomiasis, speculating on their significance.

Other chapters deal with kinetoplastids of arthropods (F.G. Wallace), lizards (V.C.L.C. Wilson and B.A. Southgate), fish (J. Lom), including lists of 149 alleged species of *Trypanosoma* and 33 of *Trypanoplasma*, and rodents (G.A.T. Targett and P. Viens), with emphasis on recent immunological work (much of it their own). Two general chapters include one on nutrition (S.H. Hutner, C.J. Bacchi and H. Baker) and one on characterisation, nomenclature and maintenance (W.H.R. Lumsden and D. Ketteridge).

Inevitably, perhaps, the choice of topics and their arrangement seems somewhat random; some deal with well known groups, presenting new information and syntheses, while others deal with parasites less often reviewed. Both groups are valuable, all contributions being authoritative, concise, comprehensive and comprehensible. Standards of editing and production are high — as, alas, is the price. There is a good general index (though the alphabetisation under the massive subheading "*Trypanosoma*" takes some getting used to), but no author index. Altogether, a volume to be recommended and either bought or coveted by all teachers or researchers of the kinetoplastids: it is sad that further volumes are not planned "at present". □

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● The second edition of *The Dynamics of the Upper Ocean*, by O.M. Phillips (for review, see *Nature*, **272**, 194; 1978), has been published by Cambridge University Press in paperback at £7.95.

● In the review of *Image Analysis, Enhancement and Interpretation*, by D.L. Missell (*Nature*, **282**, 885; 1979), the prices were incorrectly quoted, thus making the final criticisms of the reviewer unfounded. These should have read: Hardback \$60, Dfl.135; paperback \$31.75, Dfl.65.

● In the review of *Scientific Models and Man* (*Nature*, **283**, 113; 1979), the name of one of the lecturers was incorrectly quoted. This should have been: Donald Michie.

Planetary harmony

David W. Hughes

The Harmony of the World: A Realization for the Ear of Johannes Kepler's Astronomical Data from Harmonices Mundi, 1619. Gramophone record. By John Rodgers and Willie Ruff. (Sky Publishing: Cambridge, Massachusetts, 1979.) \$11.50 (including postage and packing).

KEPLER, apart from being one of the greatest, is also one of the more endearing astronomers because of his habit of including in his books a detailed account of the blind alleys he went along as well as the paths of success. He was convinced that the ways in which knowledge is acquired are as important as the knowledge itself. It's a pity more people don't think the same today.

One of Kepler's main goals in life was the

discovery of the mathematical rules underlying the progression of planetary orbital sizes, shapes and periodicities. A blind alley on the journey to this goal was the hypothesis of planetary harmony — the mathematical music of the spheres. To quote from Kepler's book *Harmonices Mundi*: "The heavenly motions are nothing but a continuous song for several voices, to be perceived by the intellect, not by the ear; a music which, through discordant tensions, through syncopations and cadenzas as it were, progresses toward certain predesigned six-voiced cadences, and thereby sets landmarks in the immeasurable flow of time".

Each planet produces a tone proportional to its angular velocity as seen from the Sun and a tonal range which depends on the orbital eccentricity. The frequency of the note is a function of the heliocentric distances. Figure 1 shows the notes that Kepler thought the planets played.

Now Kepler might have been content to perceive the heavenly chords 'by the intellect' but I have always wanted to hear

them in reality and to listen to how they change as the planets orbit the Sun. Thanks to Professor Ruff of the School of Music, Yale, and Professor Rodgers of the Geology Department of the same university, this is now possible. The first 'fixed point' of their realisation is the mean frequency of the Earth, which they set at 800 Hz — very close to the *g* (a musical expression for the *g* note about an octave and a half above middle *c*) shown in Figure 1. The second fixed point is the orbital period of Pluto (248yr) which they musically set to be 20 min 42 s. Ruff and Rodgers have updated Kepler by introducing Uranus, Neptune and Pluto. As these planets have such low frequencies they have been simulated by a series of rhythmic beats — Uranus around 10 to the second, and so on. The long playing record covers the harmony of the spheres between 27 December 1571 (Kepler's birthday) and 1835. The record also includes introductions to the planetary sounds one by one. This sequence would have been much more useful if there had been a voice announcement introducing each planet.

Scientifically the record has considerable curiosity value, and the inclusion of copious sleeve notes and a copy of an explanatory paper by Rodgers and Ruff (*American Scientist*, 67, 286-292; 1979) makes it all the more useful.

Musically the sounds have been computer generated using an IBM 360/91, and I am in deeper water. My scant musical knowledge comes from struggling through to Grade 8 piano and my musical appreciation falls into the "I know what I like" category. Bearing these facts in mind I conclude that this record put on near the end of a party would be guaranteed to clear the room in minutes flat. □

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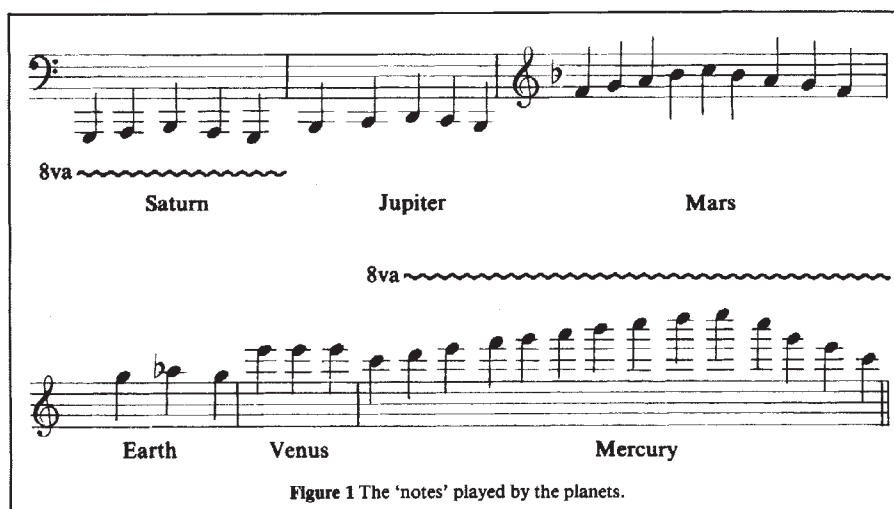


Figure 1 The 'notes' played by the planets.

Statistics in particle physics

H. Muirhead

Probability and Statistics in Particle Physics. By A.G. Frodesen, O. Skjeggstad and H. Tofte. Pp.501. (Universitetsforlaget: Bergen, Oslo and Tromsø; Columbia University Press: New York, and Guildford, UK, 1979.) Nkr.150; \$30.

WHEN I saw the name of Skjeggstad among the authors of this book, I immediately recalled a set of lectures given by him on phase space at a CERN post-graduate school in 1964. These lectures were published as a CERN yellow report and have been part of the staple diet of research students ever since. I can see this book being used in much the same way, but

not only by research students.

This book is no 'fly-by-night' progress report. It contains much solid, and at times, difficult material presented in a readable way. Although it is aimed at particle physicists the clarity of presentation should give it wider appeal. The many examples are excellent and treat real situations in particle physics. There was for me one notable omission, however, namely the treatment of the significance of a bump ('Breit-Wigner') on a background. If the bump is big and narrow and the background flat then there is no problem. If the bump is not so big and coincides with the peak in the background and one is not completely sure about the shape of the background, then the procedure becomes tricky to put it mildly.

One further thing I would like to have seen. A general warning (almost like on cigarette packages) about using common-sense, as the treatment of errors can drive

one mad. I have too often seen computer output from students containing measured quantities with nonsensical errors or χ^2 , not because the error treatment was wrong but because it had not been applied in a sensible way. One finds hints of this in the book — on page 289, for example, in discussing "pulls" the reader is advised to throw out a nonsense point from the data if he gets a bad value for χ . However, I would like to have seen a more general warning given, possibly in the preface. In reading the preface I noted that the authors paid tribute to Kendall and Stuart for their *The Advanced Theory of Statistics* (Charles Griffin: London, 1963) in three volumes, which they constantly consulted. It was also my staple stand-by 30 years ago; then it was in two volumes and had one author. I wish the present authors good fortune. □

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Facets of the fern

R.M. Lloyd

The Experimental Biology of Ferns. Edited by A.F. Dyer. Pp.657. (Academic: London, New York and San Francisco, 1979.) £37.50; \$79.

FERNS exhibit all basic phenomena of the life-cycles of vascular plants in a form which is exceptionally suitable for experimental investigation. In spite of their geological age, the group as a whole is in an active state of evolution. Although numerically small relative to the rest of the world's flora, their contribution to our understanding of the physiology of differentiation and development, genetic systems and evolution, cannot be underestimated. This multi-authored book is an attempt to put together in one volume an overview of the ultrastructural, biochemical, physiological and genetical research which has taken place during the past 40 years in experimental pteridology. All significant facets of the fern life-cycle are covered, from meiosis and spore initiation, through gametophyte morphogenesis, gametangial initiation, gametogenesis and the influence of antheridiogens, to sporophyte development, apogamy, biosystematics and experimental ecology. The book is intended to make more biologists aware of the experimental research already completed in ferns as well as their potential for further highly productive research. To facilitate this, all of the authors have quite exceptionally circumscribed the available literature in their

respective fields and have stressed not only those aspects which are in need of further work but also the currently prevalent controversial hypotheses.

Contributions of particular note are those by Peter Bell, who discusses the fern life-cycle in the conceptual framework of vascular life-cycles as a whole and presents the case for the cytological control of gametophyte sex as well as the evidence pertaining to the expression of gametophytic and sporophytic genes in spores and zygotes; Ed Klekowski, who summarises much of the evidence in support of his controversial theories on homoeologous chromosome pairing and the genetic system; J.M. Pettitt, who details our relatively meagre knowledge of spore wall structure, morphogenesis and cytochemistry; and Richard White, who effectively perpetuates the opposing arguments on the extent of developmental activity in the shoot and root apical systems. Over half of the text is devoted to research on the gametophyte generation, reflecting accurately the heavy emphasis it has received due, no doubt, to its ease of culture and manipulation. In contrast, modern ecological work in ferns is severely limited and the contribution by Chris Page adamantly exposes the critical need for further work in this area.

As this volume is technical and an excellent literature summary and as it emphasises the current sophistication and problems of experimental pteridology, it will be an important reference book for years, if not decades, to come. I, for one, am extremely pleased that it has appeared.

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Basic statistical mechanics

P. T. Landsberg

Convexity in the Theory of Lattice Gases. By R. Israel. Pp.167. (Princeton University Press: Princeton, New Jersey, and Guildford, UK, 1979.) £10.40.

By developing a series of theorems of considerable abstractness and generality, the author links together the statistical thermodynamics of lattice gases (for example using models) and the theory of convex domains. The connection between the convexity and homogeneity of thermodynamic functions is considered (a) in a finite system and (b) in the thermodynamic limit when the number of particles n and the volume v of the system both tend to infinity in such a way as to keep their ratio constant.

This book is clearly essential reading for those interested in the modern discussion

of basic statistical mechanics. It requires familiarity with the technical mathematics developed by Ruelle and colleagues. Both the concept of Hausdorff dimension and Banach space are here needed for a discussion of the Gibbs phase rule, which is treated in the sixth and last chapter of the book. New proofs concerning the existence of phase transitions are given in chapter five, by means of a modification of a theorem in pure mathematics about convex sets, which was given in 1963 by E. E. Bishop and R. R. Phelps.

To extract the physical significance of the results beyond the rather general indication given above is difficult, even if adequate space were available in this review. The job of obtaining an intuitive understanding of these detailed results is eased by the masterful Introduction which is provided by A. S. Wightman in 81 pages. Even so, much 'vulgarisation' will have to be done before this topic will find its way into the textbooks. □

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Quantitative electron microscopy

V.E. Cosslett

Introduction to Analytical Electron Microscopy. J.J. Hren, J.I. Goldstein and D.C. Joy. Pp. 601. (Plenum: New York and London, 1979.)

THE history of light optical microscopy has shown a steady progression in the use of methods for obtaining quantitative information about a specimen. Modern high-power microscopes permit measurements of absorption, refractive index, birefringence and so on, as well as morphometry. It is not surprising, therefore, that electron microscopy is following the same trend, but with greater acceleration. During the past 25 years a whole range of *In situ* analytical techniques have been developed, from crystal structure determination by microdiffraction to localised chemical analysis by X-ray excitation and energy-loss spectrometry.

The present volume presents the first comprehensive attempt to bring this battery of research tools to the attention of electron microscopists. It comprises the lectures at a 'Workshop' with the same title which was part of the annual meeting of the Electron Microscopy Society of America in August 1979. The editors succeeded in persuading the 22 co-authors to provide their manuscripts in time for the book to be printed and handed to participants when they arrived in San Antonio. Any editor of a collective work will appreciate the magnitude of this achievement, partly dependent perhaps on the use of a word processor.

We thus have available a complete review of the subject, starting with basic chapters on image formation and the electron optics of the microscope, through the instrumentation and application of the various analytical techniques, to discussion of radiation damage effects and the computer simulation of images. All the authors are expert in their particular fields, so that the numerous references are well selected and up-to-date. As acknowledged by the editors in the preface, such an enterprise undertaken at such a speed has inevitably resulted in a fair number of minor errors both technical and typographical. In view of the value of the product of their labours, however, we may accept the plea that these are 'tolerable imperfections under the circumstances', and wish them the opportunity of a second edition in which they can be corrected. □

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